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16375

Differential Agglutination of Normal and Sensitized Sheep Erythrocytes by Sera of Patients with Rheumatoid Arthritis.

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While performing complement fixation tests for rickettsialpox, the convalescent serum of a patient was observed to agglutinate the unhemolysed sheep erythrocytes. The patient, in addition to having suffered recently from the rickettsial infection, was also afflicted with rheumatoid arthritis in an active state. Further investigation revealed that the serum of this individual would agglutinate normal sheep erythrocytes to a dilution of only 1-16, whereas the same erythrocytes after being "sensitized" by two units of rabbit anti-sheep cell amboceptor were agglutinated to a serum dilution of 1-2048, a 128-fold increase in titer. A study was then made to determine under what circumstances a similar phenomenon was produced with the sera of other persons. The results of this study indicate that a 16-fold difference, or more, between the agglutination titers of normal and sensitized sheep erythrocytes, occurs almost exclusively with the sera of patients with rheumatoid arthritis, and that the magnitude of the difference in titer usually reflects the clinical activity of the disease. The present report outlines the method of performing the differential sheep cell agglutination test and describes the findings in a group of patients with rheumatoid arthritis and a variety of other diseases.

Methods and Materials. The suspensions of normal sheep erythrocytes were prepared

from fresh, defibrinated blood by washing the cells 3 times in saline (0.85% solution of sodium chloride), packing by centrifugation in a graduated tube, and resuspending in saline to make a 1.0% suspension by volume.

The suspensions of sensitized sheep erythrocytes were prepared in a manner somewhat similar to that for the Kolmer complement fixation test. Dilutions of rabbit anti-sheep cell serum ranging from 1-1000 to 1-10,000 were made in saline. Five-tenths ml of each dilution was then mixed with 0.5 ml of a 2.0% suspension of washed sheep cells and to each tube 0.1 ml of a 1-10 dilution of fresh guinea pig complement was added. The tubes were inspected after incubation in a water bath at 37°C for one hour and the highest dilution of the serum producing complete hemolysis was considered to contain one unit of hemolysin. For the agglutination test, a 2.0% suspension of the cells was mixed with an equal volume of rabbit anti-sheep serum diluted so as to contain 2 units of hemolysin. For example, if the unit of hemolysin were contained in a 1-4000 dilution of the serum, then a 1-2000 dilution would be employed for the final preparation of the sensitized erythrocytes.

The sera of the patients were obtained from freshly collected venous blood and the complement was inactivated by heating at 56°C for 30 minutes. Serial doubling dilutions of the serum were made in duplicate in 2 series of 12 tubes containing 0.5 ml of saline. To each tube of one series was added 0.5 ml of the 1.0% suspension of the normal sheep erythrocytes, and to each tube of the other series a similar amount of the 1.0% suspension of sensitized erythrocytes. The final serum dilutions were therefore 1-4, 1-8, 1-16, . . . 1-8192. (For most tests it is unnecessary to carry out serum dilutions with the normal sheep erythrocytes beyond a dilution of 1-128).

The racks containing the tubes were placed first in a water bath at 37°C for one hour and then in a refrigerator at 4°C overnight. Readings were made immediately after removing the racks from the refrigerator by flipping the tubes with the finger and estimating the degree of agglutination with the naked eye. Four-plus agglutination was indicated by a tightly-packed disc of cells and one-plus agglutination by finely granular clumping. The end-point of each titration was read as the serum dilution in the last tube showing one-plus agglutination, and the result was expressed as the reciprocal of that dilution. The agglutination titers of the individual sera for normal and sensitized sheep cells were then compared and the outcome of the test was recorded as the algebraic difference between the titers. For example, if the titer was 16 with normal erythrocytes and 1024 with sensitized erythrocytes, the result was recorded as 64.

Results. Differential sheep cell agglutination tests were performed with the sera of 110 patients. Table I shows the detailed results of the tests in representative cases, which illustrate the character of the actual findings and indicate the basis for calculating the differential agglutination titers.

In Table II are shown the results of the differential sheep cell agglutination test in the entire group of 110 patients, of whom 51 had some form of rheumatoid arthritis, 15 had rheumatic fever, 15 had arthritis other than rheumatoid or rheumatic fever, and 29 had no evidence of arthritic disease whatever.

Rheumatoid Arthritis. In 27 cases of rheumatoid arthritis with clear-cut subjective and objective evidence of active joint disease the difference between the titers of the normal and the sensitized sheep cells was never less than 16, and in 23 cases the difference in titer ranged from 32 to 512. On the other hand, in 16 cases of rheumatoid arthritis all of whom apparently were in remission, 11 gave differential titers of 16 or less, and only 5 had titers of 32 or higher.

The sera of 3 children with juvenile rheumatoid arthritis (Still's disease) were examined. One of these children, in remission, had a differential titer of 4, while the other 2, whose disease was active, gave values of 128.

The findings in 5 cases of Marie-Strumpell arthritis were of considerable interest, especially because of the questionable relation-

TABLE I.
Representative Results of Differential Sheep Cell Agglutination Test.

Clinical diagnosis	Cell suspension*	Serum dilution 4	8	16	32	64	128	256	512	1024	2048	4096	8192	Differential agglutination titer
Rheumatoid Arthritis—	NS	3+	2+	0	0	0	0	0	0	0	0	0	0	256
Active	SS	4+	4+	4+	4+	4+	4+	4+	3+	2+	1+	0	0	
Rheumatoid Arthritis—	NS	4+	4+	3+	2+	0	0	0	0	0	0	0	0	128
Active	SS	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+	2+	0	
Rheumatoid Arthritis—	NS	4+	4+	3+	1+	0	0	0	0	0	0	0	0	4
Inactive	SS	4+	4+	4+	3+	2+	1+	0	0	0	0	0	0	
Rheumatoid Arthritis—	NS	2+	0	0	0	0	0	0	0	0	0	0	0	8
Inactive	SS	4+	3+	2+	1+	0	0	0	0	0	0	0	0	
Rheumatic Fever	NS	3+	2+	1+	0	0	0	0	0	0	0	0	0	8
	SS	4+	4+	3+	2+	2+	1+	0	0	0	0	0	0	
Undifferentiated Joint	NS	4+	4+	3+	1+	0	0	0	0	0	0	0	0	2
Disease	SS	4+	4+	3+	2+	1+	0	0	0	0	0	0	0	
Infectious Mononucleosis	NS	4+	4+	4+	4+	3+	2+	1+	1+	0	0	0	0	0
	SS	4+	4+	4+	4+	3+	2+	1+	1+	0	0	0	0	

* NS = normal sheep erythrocytes.
SS = sensitized sheep erythrocytes.

TABLE II.
Differential Agglutination of Normal and Sensitized Sheep Erythrocytes by Sera of 110 Patients.

Clinical diagnosis	No. of cases	Differential agglutination titer 0	2	4	8	16	32	64	128	256	512
Rheumatoid Arthritis—Active	27					4	4	7	8	3	1
" " Inactive	16	2	1	4	1	3	2	1		2	
Juvenile Rheumatoid Arthritis (Still's Disease)	3			1					2		
Marie-Strumpell Arthritis	4		1		3						
" " " with Peripheral Joint Involvement	1								1		
Rheumatic Fever	15	1	6	1	7						
Arthritis Other than Rheumatoid or Rheumatic Fever	15	3	6	5	1						
Infectious Mononucleosis	3	2	1								
Other Disease Without Evidence of Arthritis	26	6	8	8	2	2					

ship of this disease to typical rheumatoid arthritis. Four of these patients had what we consider negative tests, with titers of 8 or less. The remaining patient had a titer of 128, and this individual was the only one with evidence of peripheral joint involvement.

Rheumatic Fever. In 15 cases of rheumatic fever, most of them active with elevated sedimentation rates and antistreptolysin titers, the sheep cell agglutination test never gave differential titers exceeding 8. Several of these patients were suffering from the adult type of rheumatic fever which is often difficult to distinguish clinically from rheumatoid arthritis.

Arthritis Other Than Rheumatoid Arthritis and Rheumatic Fever. Fifteen patients were studied in this group, with the following diagnoses: Undifferentiated arthritis of mild character, 5 cases; degenerative joint disease (osteoarthritis), 2 cases; dermatomyositis, 2 cases; lupus erythematosus disseminatus, 2 cases; and one case each of gonococcal arthritis, gout, arthritis associated with lymphogranuloma venereum, and Sudek's atrophy. None of these patients had titers exceeding 8.

Other Disease Without Evidence of Arthritis. There were 29 patients in this group, all of whom were patients on the general medical wards, with a variety of diseases none of which was associated with any arthritic involvement. Two of these patients were found to have a differential agglutination titer of 16, while the remaining 27 all had a titer of 8 or less. Three cases of infectious mononucleosis were included, which are assigned a separate place in Table II.

Correlation of Differential Sheep Cell Agglutination with Erythrocyte Sedimentation Rate and Streptococcal Agglutination. Erythrocyte sedimentation rates were performed by the Westergren method in nearly all patients at about the same time that blood was drawn for the differential sheep cell agglutination test. As might be expected, most of the patients with active rheumatoid arthritis who had high differential agglutination titers also had high sedimentation rates. However, many of the other patients exhibited equally high sedimentation rates but had differential agglutination titers of less than 16. Consequently, it would appear that there is no correlation between the height of the sedimentation rate and the results of the differential sheep cell agglutination test.

It is well established that many patients with rheumatoid arthritis, especially those with active, progressive disease, possess an antibody in their serum which will agglutinate Group A hemolytic streptococci.[1,2] Indeed, the streptococcal agglutination test is employed as an adjunct in the diagnosis of rheumatoid arthritis. Streptococcal agglutination tests were performed in all of the cases of rheumatoid arthritis and the results were compared with those of the differential sheep cell agglutination test. In general, the patients with active rheumatoid arthritis with differential agglutination titers of 16 or higher also gave positive streptococcal agglutination tests. However, there was no correlation between the positivity of the two reactions and, in addition, several cases of active rheumatoid arthritis with high differential sheep cell titers had either negative or doubtful streptococcal agglutination tests. It therefore seems probable that the substance in the serum responsible for the differential agglutination of normal and sensitized sheep erythrocytes is different in nature from that responsible for the agglutination of Group A hemolytic streptococci.

Recent reports have suggested that the agglutination of streptococci by the sera of patients with rheumatoid arthritis is a nonspecific or panagglutinative effect, since the same sera may also agglutinate collodion particles.[3] In our experience we have found no direct relationship between these reactions.[4] Furthermore, with 10 sera selected at random from the present group of patients, the differential sheep cell agglutination titer bore no relation to the agglutination of collodion

[1] Nicholls, E. E., and Stainsby, W. J., *J. Clin. Invest.*, 1931, **10**, 323.

[2] Dawson, M. H., Olmstead, M., and Boots, R. H., *J. Immunol.*, 1932, **23**, 187.

[3] Wallis, A. D., *Am. J. Med. Sci.*, 1946, **212**, 713.

[4] Lipman, M. O., Coss, J. A., Jr., and Ragan, C., in preparation.

particles, either plain, coated with nutrient broth, or coated with the broth filtrate of cultures of hemolytic streptococci.

The Serum Fraction Containing Agglutinin for Sheep Cells. Differential sheep cell agglutination tests were carried out with the electrophoretically separated beta-gamma globulin fractions of the serum from cases of active and inactive rheumatoid arthritis and the results are shown in Table III. It will be observed that the differential agglutination of normal and sensitized erythrocytes by the purified globulin fractions resembles that seen with the whole serum of similar cases.

Discussion. When fresh, heat-inactivated human sera are tested for their ability to agglutinate both normal sheep erythrocytes and sheep erythrocytes "sensitized" by their exposure to small amounts of rabbit anti-sheep cell serum, the sensitized erythrocytes are almost always agglutinated to a higher titer than are the normal erythrocytes. With the sera of patients suffering from rheumatic fever, various arthritic disorders other than rheumatoid arthritis, and diseases unassociated with joint involvement, the difference between the agglutination titers of normal and sensitized sheep erythrocytes is usually of a low order and thus far has not been observed to exceed an algebraic difference of 16. These findings are in marked contrast to those with the sera of patients suffering from rheumatoid arthritis, especially when the disease is in an active state. In such patients the differential titer is never lower than 16—the upper limit of the "normal" values—and is usually considerably higher. On the other hand, in patients with clinically inactive rheumatoid arthritis, the differential agglutination titer falls within normal limits in the majority of cases. The conclusion therefore seems valid that one of the unique features of active rheumatoid arthritis is the presence in the serum of a substance which will agglutinate sensitized sheep erythrocytes to a markedly higher titer than it will agglutinate normal sheep erythrocytes. This substance occurs in the globulin fraction of the serum and does not seem to be associated with factors which are responsible for alterations in the sedi-

TABLE III.

Differential Sheep Cell Agglutination Tests with Electrophoretically Separated Beta-Gamma Globulin Fractions of Sera from Patients with Active and Inactive Rheumatoid Arthritis.

Clinical diagnosis	Cell suspension	Dilution of globulin fraction*							Differential agglutination titer
		10	20	40	80	160	320	640	1280	
Inactive Rheumatoid Arthritis	NS	++++	++++	+	0	0	0	0	0	4
	SS	++++	++++	+++	++	+	0	0	0	
Active "	NS	0	0	0	0	0	0	0	0	128
	SS	++++	++++	++++	+++	+++	++	+	0	

* Dilutions expressed as final dilutions of original serum.

mentation rate of eryt... glutination of Group A ... cocci. It is probably unrela... glutinin reaction of Bordet ... complement is not required ... The precise nature of the sub... significance with regard to the ... state remain to be determined. I... the present observations indica... sheep cell differential agglutinatio... be of value in determining the acti... tus of patients suffering from rheumatoid ... itis.

[5] Bordet, J., and Gay, F. P., *Ann. Inst. Pasteur*, 1906, **20**, 467.

...t may also pro... helpful as an aid in the differential ... between rheumatoid arthritis and ... iseases with arthritic manifestations, su... as rheumatic fever.

Summary. A ...fferential sheep cell agglutination te... s described wherein the agglutination ti... of human serum for normal and sensitized ...heep erythrocytes are compared. High dif...rential titers have been found to occur almos... solely with the sera of patients suffering from active rheumatoid arthritis. Some of the features of this serological phenomenon are pointed out, together with its possible practical applications.

16376

Phospholipid Metabolism in Diabetes: Turnover Rate of Plasma Phospholipids in Completely Depancreatized Dogs.

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The increased amounts of fatty acids that appear in both liver and plasma of dogs deprived of all islet tissue indicate that diabetes in this animal is characterized by a severe disturbance in fat metabolism.[1,2] It is shown here, however, that the turnover of plasma phospholipids is not altered in the diabetic dog. Apparently, plasma phospholipids do not participate in the redistribution of fat that occurs in the diabetic dog.

Experimental. Depancreatized Dogs. After complete excision of the pancreas the dogs were injected with insulin and fed twice daily a mixture containing lean meat, raw pancreas, sucrose, and vitamin supplements.[3]

Synthesis of Labeled Plasma Phospholipids. To provide labeled plasma phospholipids, 2-3 millicuries of radioactive phosphate were administered by stomach tube to fasted normal dogs. Twenty-four hours later the dogs were bled, the heparinized blood centrifuged, and the plasma containing the radioactive phospholipid separated.

Methods of Analyses. Phospholipid P^{31} and phospholipid P^{32} were determined in plasma in a manner described elsewhere.[4] The method used for determination of plasma total-fatty acids has also been recorded elsewhere.[5] At the end of the period of observation, the whole liver was excised, thoroughly ground, and analysed for its total-fatty acid content.[6]

Experiment 1. 50 cc of plasma containing labeled phospholipids were injected intravenously into a normal and a depancreatized dog, the latter of which had been deprived of insulin but not food for 3 days. Samples of blood were removed from both dogs at several intervals and the plasma analysed for

* Fellow of the American Cancer Society (recommended by the Growth Committee).

[1] Gibbs, G. E., and Chaikoff, I. L., *Endocrinology*, 1941, **29**, 885.

[2] Bloor, W. R., Gillette, E. M., and James, M. S., *J. Biol. Chem.*, 1927, **75**, 6.

[3] Montgomery, M. L., Entenman, C., and Chaikoff, I. L., *Am. J. Physiol.*, 1944, **141**, 216.

[4] Zilversmit, D. B., Entenman, C., Fishler, M. C., and Chaikoff, I. L., *J. Gen. Physiol.*, 1943, **26**, 333.

[5] Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, 1934, **106**, 267.

[6] Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, 1935, **108**, 201.

TAI

Turn ma Phospholipid

Dog		Body kg
P-70	Normal	10
R-70	Depancreatized witho sulin	6

Injected plasma contain 5,800 r.u. phosp P32 per cc.

At the end of the period of observation, 586 mg % of total fatty acids and 12 mg % 12% fatty acids.

ctive and

ntial tion

betic Dog.

lipid ne,	Plasma phospholipid turnover rate, mg phosphorus per hr
	6.9
3	7.9

cc and 2,480 r.u. acid-soluble

ne depancreatized dog contained phosphorus. Its liver contained

total-fatty acids, phospholipid P^{31}, and phospholipid P^{32}. The results are recorded in Table I. In Fig. 1 a semi-log plot has been made for the values of plasma phospholipid P^{32} against time.

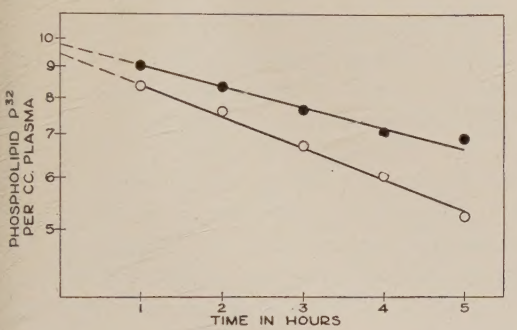

FIG. 1.

The disappearance of intravenously injected labeled phospholipids from the circulation (semi-log plot).

● Normal dog.

○ Depancreatized dog R-70.

In a previous communication[4] it was shown

$$\frac{x}{f} = \frac{cr}{f} e^{-\frac{p}{r}t} \qquad (A)$$

in which x = amount of phospholipid P^{32} present in the entire circulating fluid

f = amount of circulating fluid

c = a constant

r = total amount of phospholipid phosphorus present in the entire circulating fluid

p = rate of phospholipid phosphorus turnover

t = time after injection of phospholipid P^{32}.

Since the turnover time† (t_t) equals $\frac{r}{p}$ we can also write

$$\frac{x}{f} = \frac{cr}{f} e^{-\frac{t}{t_t}} \qquad (B)$$

$$\text{or} \quad \ln \frac{x}{f} = \ln \frac{cr}{f} - \frac{t}{t_t} . \qquad (C)$$

The turnover time (t_t) may conveniently be obtained from Fig. 1 or 2 as follows: At half-time ($t_{1/2}$) the phospholipid P^{32} per cc plasma ($x_{1/2}$) is exactly half that at zero time (t_o). Hence from equation C it follows that

$$\ln \frac{x_o}{x_{\frac{1}{2}}} = \ln 2 = \frac{t_{\frac{1}{2}}}{t_t} \qquad (D)$$

$$\text{or} \quad t_t = 1.44\ t_{\frac{1}{2}} . \qquad (E)$$

The half time can be read from the curves in Fig. 1 and 2 and the turnover time calculated by means of equation E. The turnover rate can next be calculated from the relation:

$$t_t = \frac{r}{p} \qquad (F)$$

The total amount of phospholipid P^{31} present in the entire circulating fluid, *i.e.*, r, can be found if the circulating volume (f) and the concentration of phospholipid P^{31} in plasma ($\frac{r}{f}$) are known. At "zero time" the amount of phospholipid P^{32} present in the circulation ($= x_o$) is equal to the amount of phospholipid P^{32} injected. The circulating volume can be found by dividing the amount of phospholipid P^{32} injected by the concentration of phospholipid P^{32} in plasma at zero

† Turnover time of plasma phospholipids is the time required for the formation of an amount of phospholipid equal to that present in plasma.

TABLE II.
Turnover of Plasma Phospholipids in Depancreatized Dogs Maintained With and Without Insulin.

		With insulin*				Without insulin†				
Dog.	Body wt, kg	Plasma phospholipid P31, mg %	Plasma total fatty acids, mg %	Phospholipid turnover time, hr	Phospholipid turnover rate, mg phosphorus per hr	Plasma phospholipid P31, mg %	Plasma total fatty acids, mg %	Liver total fatty acids, % wet wt	Phospholipid turnover time, hr	Phospholipid turnover rate, mg phosphorus per hr
A	5.8	23	737	6.9	12	21	749	12.6	15.1	5.2
B	9.8	16	480	8.5	15	19	894	16.0	10.8	12
C	13.0	18	369	6.3	21	29	958	13.1	9.4	18

* Injected plasma contained 4,520 r.u. phospholipid P32 per cc and 2,560 r.u. acid-soluble P32 per cc.
† Injected plasma contained 13,700 r.u. phospholipid P32 per cc and 10,200 r.u. acid-soluble P32 per cc.

time ($\frac{x_o}{f}$). The value for $\frac{x_o}{f}$ can be read from the intersection of the extrapolated curve with the "Y" axis.

From Table I it is apparent that the turnover rate of plasma phospholipid in the diabetic dog did not differ much from that of the normal. The values obtained for the normal dog are in good agreement with those reported earlier.[4]

Experiment 2. In the next experiment the turnover of plasma phospholipid was compared in the *same* depancreatized dog (1) while it was under insulin control and (2) 4 days after insulin injections had been discontinued. The results are recorded in Table II and Fig. 2.

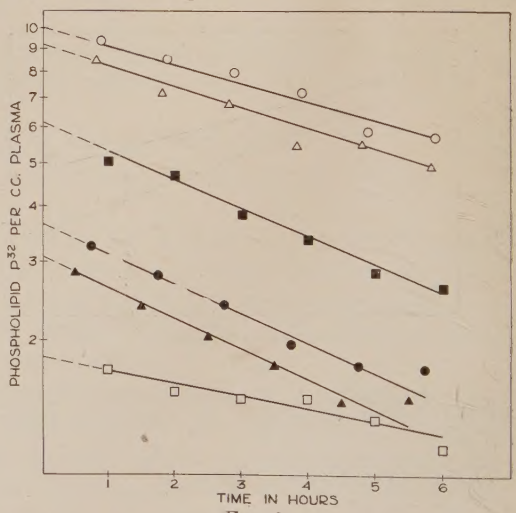

FIG. 2.

The disappearance of intravenously injected labeled phospholipids from the circulation (semi-log plot).

Dog	A	B	C
With insulin	■	●	▲
Without insulin	□	○	△

Discussion. The discontinuation of insulin administrations in the 4 depancreatized dogs resulted in an accumulation of 12-16% fatty acids in their livers (Table II). A transfer of fat from depot to liver has been suggested as the mechanism by which this type of fatty liver develops.[7] It can be calculated that an average of approximately 400 mg of fatty acids were deposited per hour in these livers. If phospholipids serve as vehicles for carrying this extra amount of fatty acids to the

[7] Best, C. H., *Am. J. Digest. Dis.*, 1946, **13**, 155.

liver, then the normal turnover of plasma phospholipid phosphorus would have been increased by an additional $\frac{520}{5.0} \times 31$ or 24 mg per hour.‡ The results obtained here clearly demonstrate that such an increase in the turnover of plasma phospholipid P did not occur. Hence, it would appear that plasma phospholipids do not participate in the disturbance in fatty acid metabolism encountered in the diabetic state.

The view that phospholipids are concerned with fatty acid transport is widely held today. Such a function for phospholipid, however, has received no support from several types of studies carried out recently in this laboratory:

In the diabetic dog there is reason to believe that considerable amounts of fat migrate from depot to liver. Yet, as noted above, the rate of turnover of plasma phospholipid in this condition was no greater than normal.

In dogs in which the liver is excised the concentration of plasma phospholipids remains constant, and their turnover practically stops.[8] This finding and other evidence show not only that plasma phospholipids are synthesized in the liver, but that this organ is the only one concerned with their utilization. These studies thus fail to support the concept that fatty acids are transported between liver and other organs via phospholipids.

Recent unpublished observations dealing with the mechanism of action of choline[9] are also pertinent here. It was shown that choline does not affect the turnover rate of plasma phospholipids, but that it does stimulate the turnover of lecithin within the liver itself. This seems to indicate that phospholipids, while mediating the lipotropic action of choline, do not bring this about by increasing the transport of fatty acids.

Finally, mention should be made of the studies on the turnover rate of the small intestine's phospholipids in the fasted and fat-fed dog and rat.[10] Here again it appeared that phospholipids are not instrumental in the transport of fatty acids across the intestinal wall.

Summary. The rate of turnover of plasma phospholipids is not increased above normal in the diabetic state. This and other evidence reviewed here fails to support the concept that phospholipids are agents for the transport of fatty acids.

‡ In the phospholipid molecule 520 mg of fatty acids are associated with 31 mg of phosphorus.

8 Entenman, C., Chaikoff, I. L., and Zilversmit, D. B., *J. Biol. Chem.*, 1946, **166**, 15.

9 Zilversmit, D. B., Entenman, C., and Chaikoff, I. L., in press.

10 Zilversmit, D. B., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, 1948, **172**, 637.

16377

Administration of Chloromycetin to Normal Human Subjects.

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The need for information on the results of administration of Chloromycetin to normal human beings became evident shortly after the chemotherapeutic properties of this antibiotic were first noted;[1,2] this became imperative recently when an opportunity presented itself for a field trial of the drug in the treatment of patients with typhus fever.[3]

1 Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

2 Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A., and Burkholder, P. R., *Science*, 1947, **106**, 417.

3 Smadel, J. E., León, A. P., Ley, H. L., Jr., and Varela, G., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 12.

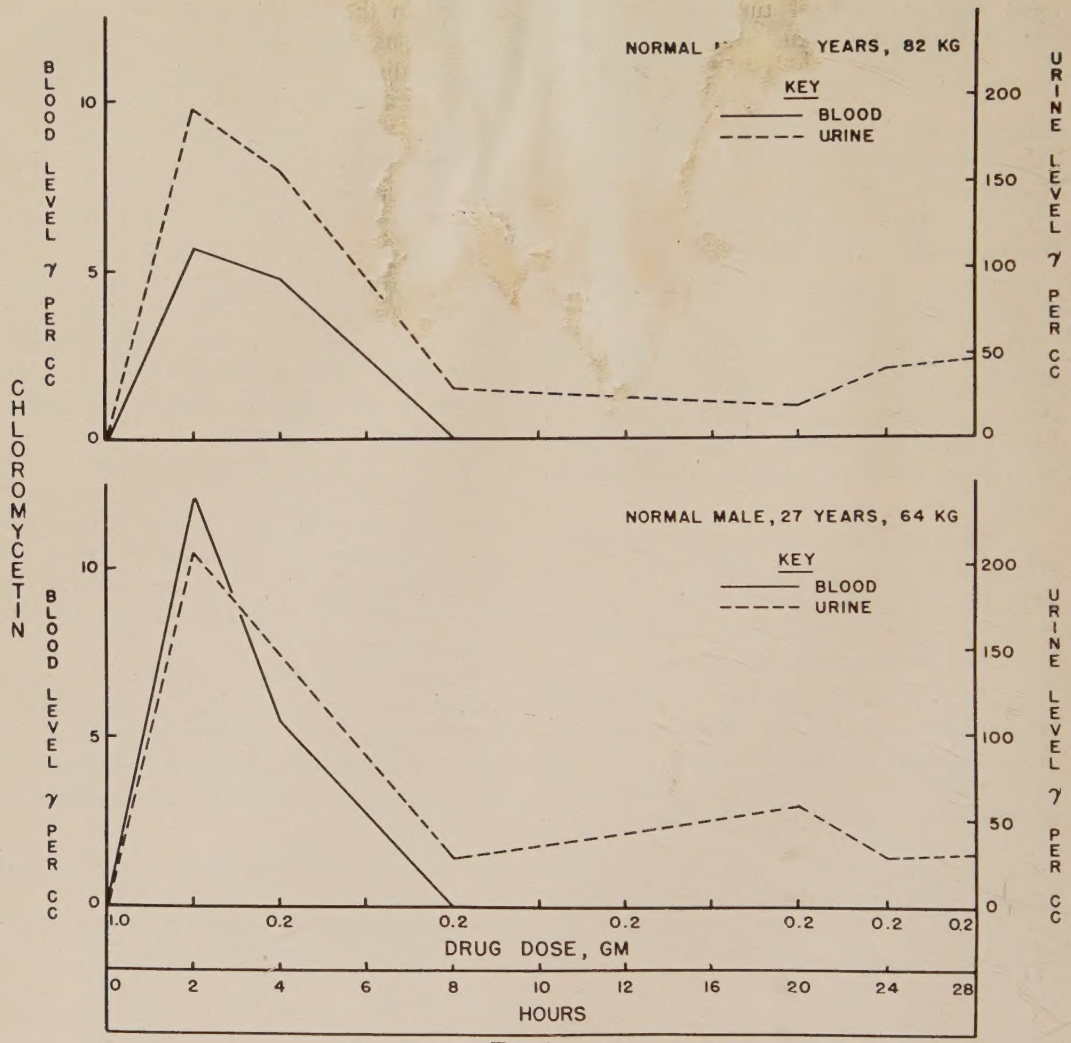

FIG. 1.

The present report summarizes the observations on three volunteers who were treated with Chloromycetin.

Materials and Methods. The oral route was selected for administration of Chloromycetin to volunteers not only because of its great convenience but also because of experimental evidence which indicated that the drug was effective by this route in treating infected mice.[1] Tablets containing 0.1 g amounts of crystalline Chloromycetin were supplied for the trial by the Research Laboratories of Parke, Davis and Company.

Levels of Chloromycetin in blood and urine were determined in samples collected at intervals throughout the study.* A modification of the method of Joslyn and Galbraith[4] which employs inhibition of growth of *Shigella paradysenteriae* (*Sonne*) was used for these microbiological assays. Blood and urine specimens taken for such assays were obtained in the following sequence: the patient voided, was bled, and then the urine sample for assay was collected within a few minutes. In addi-

* The authors wish to thank Miss A. B. Cruise who assisted with the Chloromycetin assays.

[4] Joslyn, D. A., and Galbraith, M., personal communication, 24 October, 1947.

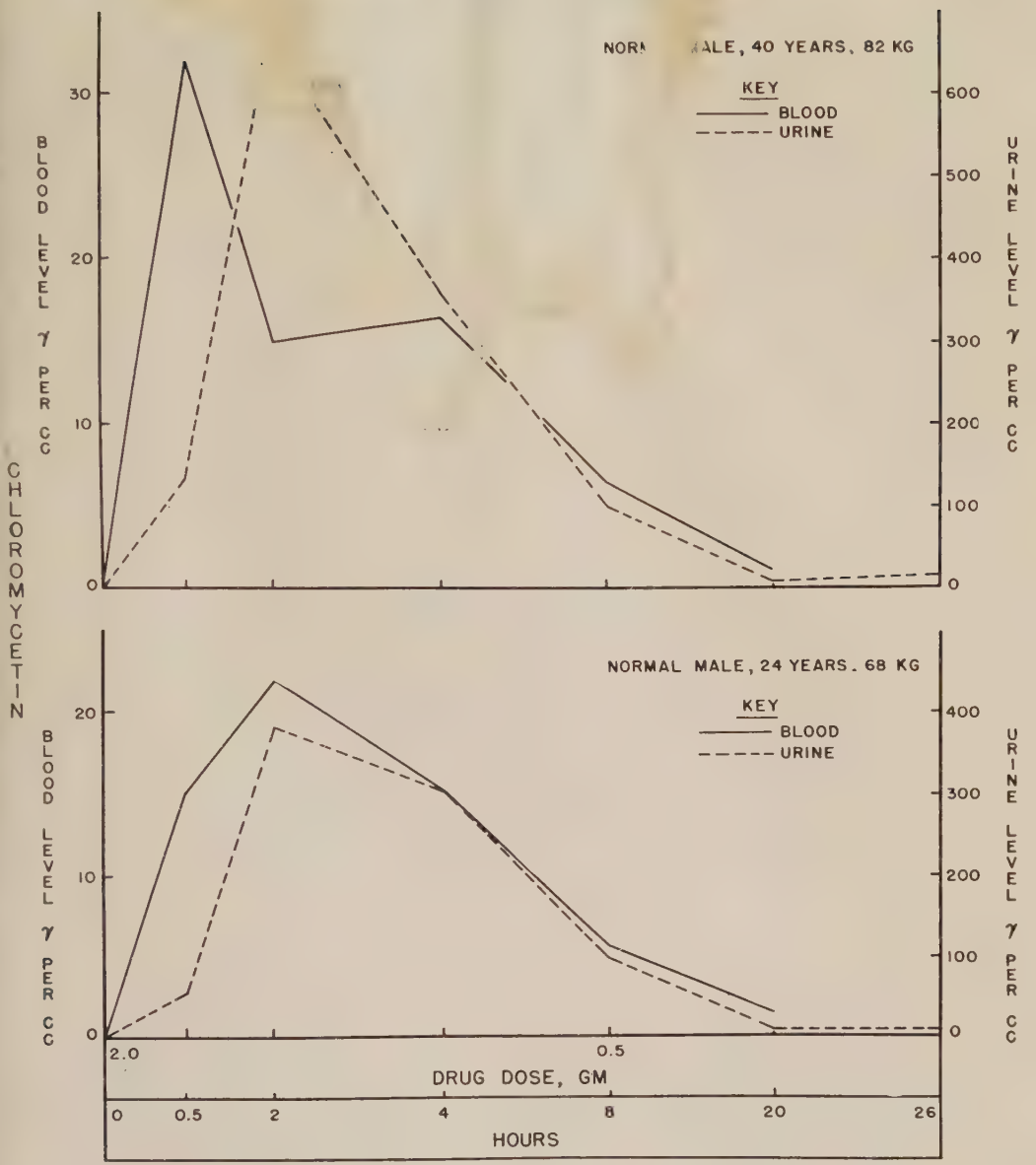

FIG. 2.

tion, urine was collected for 24-hour periods in order to permit estimation of the total daily urinary excretion of Chloromycetin. The hemoglobin levels, red and white blood cell counts, and differential leukocyte counts were obtained by the usual clinical laboratory methods.

Results. In the first test 2 normal male subjects received a prolonged course of Chloromycetin beginning with a single dose of 1.0 g which was followed by 1.0 g daily for 10 days; the latter was given in divided doses of 0.2 g every 4 hours except at 4:00 a. m. In a second test one of the volunteers from the first experiment and another subject received an initial dose of 2.0 g followed in 8 hours by a single 0.5 g dose.

Values for the levels of Chloromycetin in the blood and urine of the volunteers in the first test are given for the early portion of

the course of treatment in Fig. 1. The peak values for both blood and urine were recorded for the first specimen collected after the initial dose, *i.e.*, at 2 hours. Subsequently, the blood levels steadily fell in both subjects and detectable amounts of drug were not demonstrable at 8 hours or thereafter. The urine levels of drug were approximately 200 γ/cc at 2 hours; they fell to approximately 50 γ/cc at 8 hours and remained at about this level for the next 10 days of treatment. During and immediately following the test no significant changes occurred in the blood of either subject as revealed by determination of hemoglobin, erythrocytes and leukocytes and by examination of blood smears. Urinalysis on the third day of therapy showed no abnormalities.

Data on the volunteers who received an initial dose of 2.0 g of Chloromycetin plus 0.5 g 8 hours later are given in Fig. 2. Relatively high levels of drug were found in the blood of both volunteers only 30 minutes after the antibiotic was first given by mouth; furthermore, at this time drug was already present in the urine in appreciable amounts. The blood levels were still well above 10 γ/cc at 2 hours and were above 5 γ/cc at the end of 8 hours. Urine levels were at their maxima at 2 hours reaching values of 670 and 730 γ/cc, respectively, in the subjects. The amounts diminished rapidly in successive samples and dropped to about 10 γ/cc at 8 hours.

Approximately 10% of the total amount of Chloromycetin given daily was recovered in an active form in the urine of both groups of volunteers.

No symptoms or signs of toxic effects attributable to the drug were observed by the 3 volunteer physicians either during the treatment or subsequently.

Conclusion. These limited trials indicate that Chloromycetin can be given orally to normal adult males in single doses of 2.0 g, or in daily doses of 1.0 g for 10 days without untoward reactions. The presence of appreciable amounts of drug in the blood and urine of volunteers 30 minutes after oral administration of Chloromycetin indicates that the antibiotic is absorbed rapidly from the gastrointestinal tract of man. Excretion or inactivation of the drug occurs rather rapidly, hence, in order to maintain appreciable levels of the antibiotic in the blood, frequent administration of the drug is indicated.

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Chloromycetin in the Treatment of Patients with Typhus Fever.

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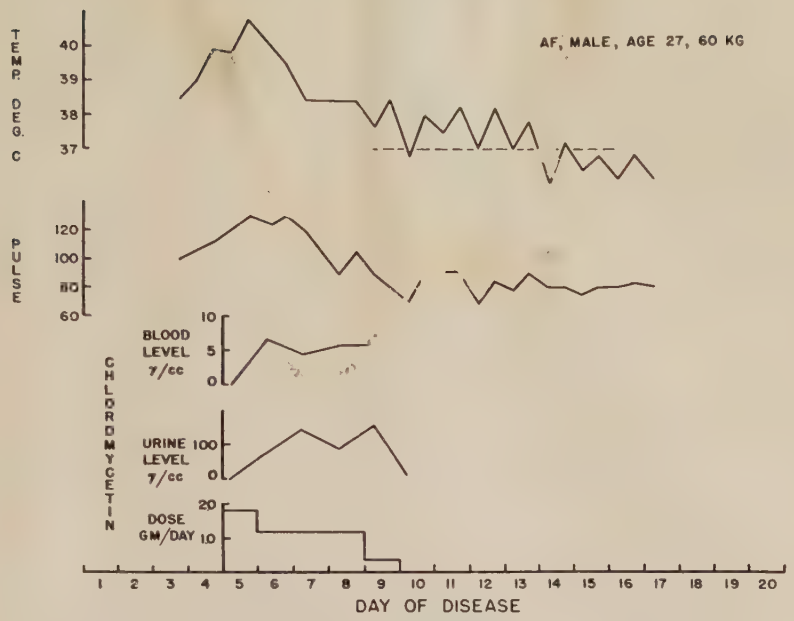
Recent reports indicate that Chloromycetin is effective in the treatment of experimental infections caused by *R. prowazeki*[1,2] and *R. mooseri* as well as other rickettsial and viral agents.[2] Data reported elsewhere[3] show that Chloromycetin can be given to normal men without untoward effects and that the levels of drug in the blood and urine of treated persons can be followed. This paper summarizes the information gained from the study of a small group of patients with typhus fever who were treated with Chloromycetin.

Materials and Methods. The patients studied in the current investigation were hos-

[1] Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A., and Burkholder, P. R., *Science*, 1947, **106**, 417.

[2] Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

[3] Ley, H. L., Jr., Smadel, J. E., and Crocker, T. T., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 9.



RASH		00	++	++	+	+	0	0	0
WBC (X 1000)		5.7	3.5	3.6	2.9	6.9		
HBG (GM)		12.2		9.8	9.5	9.5		
WEIL	OX 19	80		2560		5120		5120
FELIX	OX 2	0		0		40		320
RICK.	EPIDEMIC	0		160		1280		10240
AGGLN.	MURINE	0		40		320		2560
RICK.	EPIDEMIC	0		0		10		1280
C.F.	MURINE	0		0		0		0

FIG. 1.

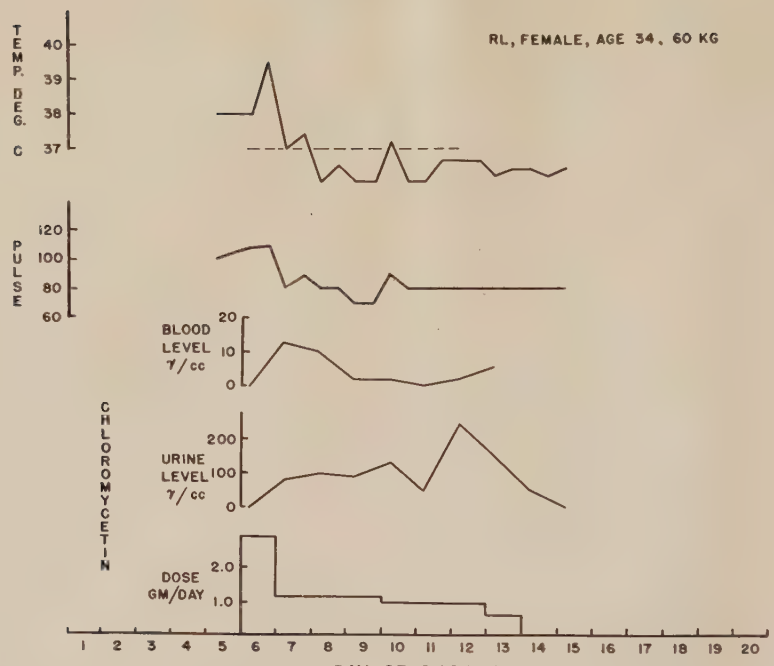
pitalized in Mexico, D.F.* Crystalline Chloromycetin prepared in 0.1 g tablets for oral administration was supplied by the Research Laboratories of Parke, Davis and Company. Chloromycetin levels in the blood and urine of patients were determined by a modification of the method of Joslyn and Galbraith.[4] Specific rickettsial complement-fixation and agglutination tests were performed according to the standard procedure of the Army Medical Department Research and Graduate School.[5] The results of the Weil-Felix tests recorded on the graphs were done by the technique employed at the School;[5] comparative studies in which sera were tested with proteus antigens prepared at the School and at the Institute by the techniques employed in both laboratories gave comparable results. León's technique,[6] for the demonstration of specific typhus antigen in the urine of early typhus patients, was employed in two of the treated patients and

* The authors wish to thank Dr. Samuel Morones, Chief of the Infectious Disease Service, Hospital General, Dr. F. Lopez Clares, Chief of the Infectious Disease Service, Hospital del Niño, and Dr. Luis Mendez, Chief, Infectious Disease Hospital, Seguro Social, for their interest in the present work and for permission to report the observations made on their wards.

4 Joslyn, D. A., and Galbraith, M., personal communication, 24 October, 1947.

5 Plotz, H., "The Rickettsiae," chapter 32 in *Laboratory Methods of the United States Army*, edited by Simmons, J. S., and Gentzkow, C. J., Lea and Febiger, Philadelphia, 1944, pp. 559-578.

6 León, A. P., *Rev. Inst. Salub. y Enf. Trop.*, 1942, **3**, 201.

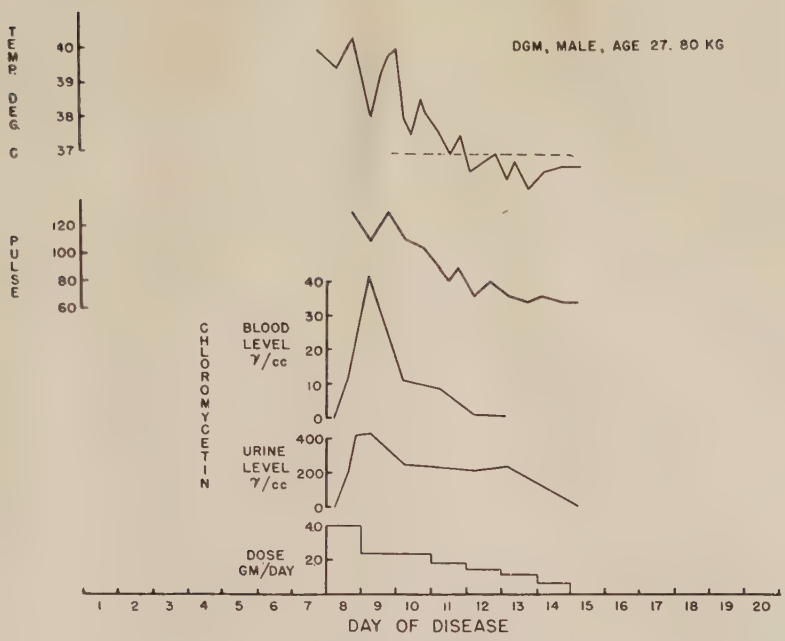


RASH			++	++	++	+	+	0	0	0			
LEONS TEST			Pos (1/8)										
WBC (X 1000)			12.4	10.4	7.6				7.3		10.7		
HBG (GM)			12.1	11.1	11.5				12.3			13.2	
WEIL	OX 19	40				80	80	160	160	160		160	
FELIX	OX 2	0				0	0	0	0	0		0	
RICK.	EPIDEMIC	80				640	1280		1280			1280	
AGGLN.	MURINE	40				80	160		320			160	
RICK.	EPIDEMIC	80				640	1280		1280	1280+		640	
C.F.	MURINE	5				80	160		320	160		160	

FIG. 2.

found to be positive in each instance.

Results. Five patients with typhus fever were treated with Chloromycetin. More complete and definitive information was obtained on the 3 adults than on the 2 children; therefore, each of the former will be discussed in some detail while the latter will be presented briefly. Data on the first patient A.F., are given in Fig. 1. This man received an initial dose of 1.0 g of drug by mouth on the morning of the fifth day of illness and subsequently was given 0.2 g every 4 hours throughout the next 3 days and part of the fourth day. The levels of Chloromycetin in the blood and urine of this patient ranged around values of 5 and 100 γ per ml, respectively. During the course of therapy the white blood cells diminished progressively from 5,700 on the fifth day of disease to 2,900 on the eighth day. At this point ideas were entertained about the possible relationship between the drug and the leukopenia. These were dismissed on the ninth day when the patient's white cell count rose to 6,900 while he was still on therapy. There was a reduction in the hemoglobin content of the blood between the fifth and seventh days, however, it was assumed that the patient actually had a moderate anemia at the onset of his illness and that the initial value of 12.2 g was dependent upon hemoconcentration during the period of high fever. The serological data presented in Fig. 1 indicate that this patient had epidemic typhus. It is difficult to say how much patient A.F. was

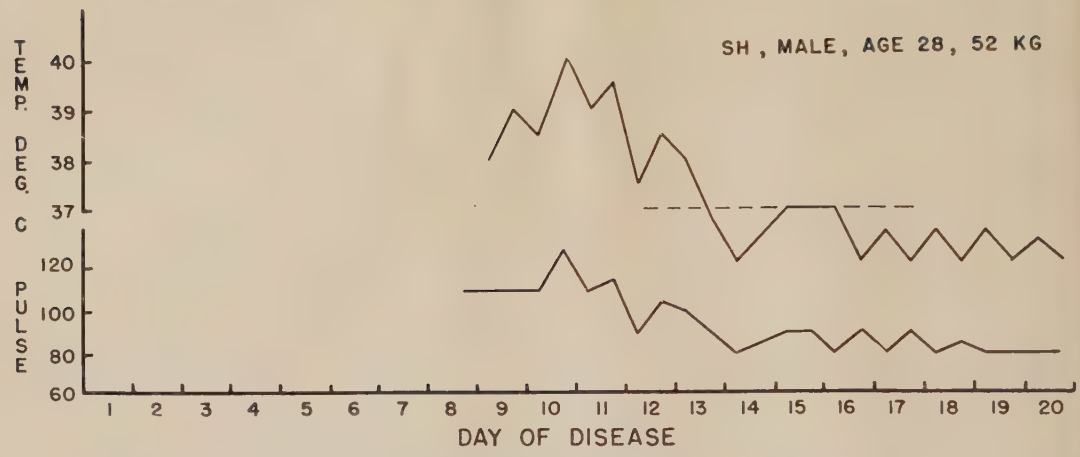


DELIRIUM					++	++	++	0	0	0									
RASH					+++	+++	+++	+++	+++	++	+	0	0	0					
WEIL	OX 19	640			5120		5120							5120					
FELIX	OX 2	0			80		80							80					
RICK.	EPIDEMIC	40					5120							10240					
AGGLN.	MURINE	0					640							1280					
RICK.	EPIDEMIC	0					0							80					
CF.	MURINE	0					0							0					

FIG. 3.

benefited by treatment with Chloromycetin. In retrospect, it was the impression of the group that patient A.F. had been treated with doses of drug which were too small and that the antibiotic had been given for too short a period of time.

Patient R.L., whose record is summarized in Fig. 2, lived with patient A.F. Her disease, characterized initially by feverishness and headache, began on the day that patient A.F. was hospitalized. She had a cutaneous rash when admitted on the fifth day of her illness. León's test performed with a sample of her urine taken on the morning of the sixth day gave a positive reaction; a Weil-Felix test on serum obtained at this time was positive at a dilution of 1/40. The patient was given 2.0 g of Chloromycetin at noon on the sixth day. At 1:00 p. m. she vomited and was promptly given 0.5 g of Chloromycetin. This was followed by administration of 0.2 g every 4 hours until the morning of the 13th day of her illness. The blood level of Chloromycetin reached 11.5 γ per ml on the morning of the second day of treatment and remained in this range the next day, but subsequently dropped to a value of about one γ per ml, see Fig. 2. The urine level ranged around 100 γ per ml for several days but reached 220 γ per ml on the seventh day of treatment. The abrupt return of the patient's temperature and pulse to the normal range following administration of Chloromycetin at first led to the suspicion that she did not have typhus, but the serological data subsequently indicated that this woman was infected with *R. prowazeki*. Other clinical abnormalities abated more slowly than the fever; thus, on the morning of the seventh day when the temperature was 37°C, the rash remained unchanged, the patient

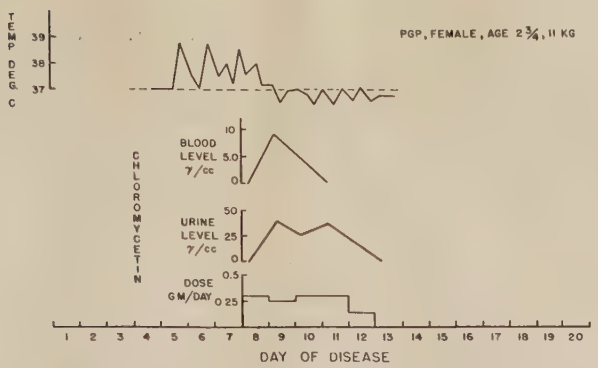


RASH		++	++	++	+	0	0	0		
WEIL FELIX	OX 19	160							160	320
	OX 2	0							40	40
RICK. AGGLN.	EPIDEMIC	2560							5120	10240+
	MURINE	320							1280	2560
RICK. C.F.	EPIDEMIC	160							160	320
	MURINE	0							0	0

FIG. 4.

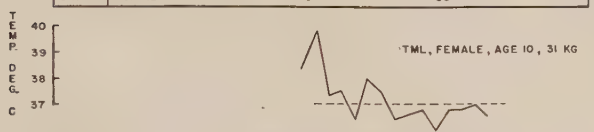
complained of headache and insomnia, and she still had flushed facies and conjunctival injection, as well as slight tremor of the tongue. The following day the patient was much improved and on the ninth day after onset she had no complaints, the rash was fading, and the conjunctival injection as well as the tremor and dryness of the tongue were gone. On the 12th day the patient was cheerful and sat up in bed. She was discharged from the hospital on the 15th day after onset.

Patient D.G.M. was admitted to the hospital on the seventh day of an illness which appeared typical of typhus fever. He was extremely ill with a fever of 40°C and had an extensive, discrete macular rash which was not yet hemorrhagic. The Weil-Felix test at this time was positive at a dilution of 1/640. At 1:00 p. m. on the eighth day of illness the patient was given 1.5 g of Chloromycetin by mouth, this was repeated at 2:00 p. m. Subsequently he received 0.2 g every 2 hours day and night until the morning of the 11th day at which time the dosage was reduced to 0.2 g every 3 hours. The following day the drug was further reduced to 0.2 g every 4 hours and the drug was discontinued on the morning of the 14th day. The blood level of Chloromycetin rose rapidly and reached a value of 40.5 γ per ml at 8:00 a. m. on the morning of the second day of therapy; similarly the urine level had risen progressively and reached a value of 400 γ per ml at this time, (See Fig. 3.) During the next few days the blood level of drug ranged between 10 and 5 γ per ml and the urine around a value of 100 γ per ml. Although the temperature dropped to 38°C on the morning after therapy was begun, the patient showed no other obvious evidence of improvement; the semidelirium continued, the rash had become petechial in some areas, the conjunctivae were still markedly injected, and fine tremors of the tongue and extended fingers continued unabated, furthermore, that afternoon the temperature returned to 40°C. On the morning of the tenth day the temperature was 37.5°C, the cutaneous lesions were unchanged and delirium still persisted. Despite the pos-

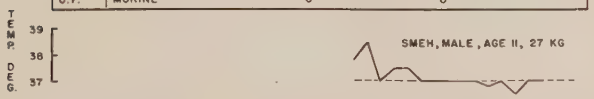


WBC (X 1000)			9.2		
HBG (GM)			8.5		
WEIL	OX 19	5120+	5120+	5120+	5120+
FELIX	OX 2	0	80	80	80
RICK.	EPIDEMIC	160	320	320	160
AGGLN	MURINE	1280	2560	2560	5120
RICK.	EPIDEMIC	0	0	0	0
C.F.	MURINE	0	0	0	80

RASH		+	+ +	0 0 0
WEIL	OX 19	1280		2560
RICK.	OX 2	320		320
RICK.	EPIDEMIC	80		640
AGGLN.	MURINE	1280		2560
RICK.	EPIDEMIC	0		640
C.F.	MURINE	0		80



WEIL	OX 19	2560	5120
FELIX	OX 2	160	640
RICK.	EPIDEMIC	40	320
AGGLN.	MURINE	320	5120
RICK.	EPIDEMIC	0	0
C.F.	MURINE	0	0



WEIL	OX 19	320	160
FELIX	OX 2	40	0
RICK.	EPIDEMIC	320	320
AGGLN.	MURINE	5120	2560
RICK.	EPIDEMIC	0	0
C.F.	MURINE	0	80

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

DAY OF DISEASE

FIG. 5.

sibility that the drug therapy might be contributing to the delirium, Chloromycetin was continued. The next day the patient's temperature was normal and he was lucid; therefore, it is assumed that the drug had not played any role in producing the patient's confused mental state. The improvement from the 11th day was obvious. It was the impression of the group that this patient represented an extremely severe case of epidemic typhus, in

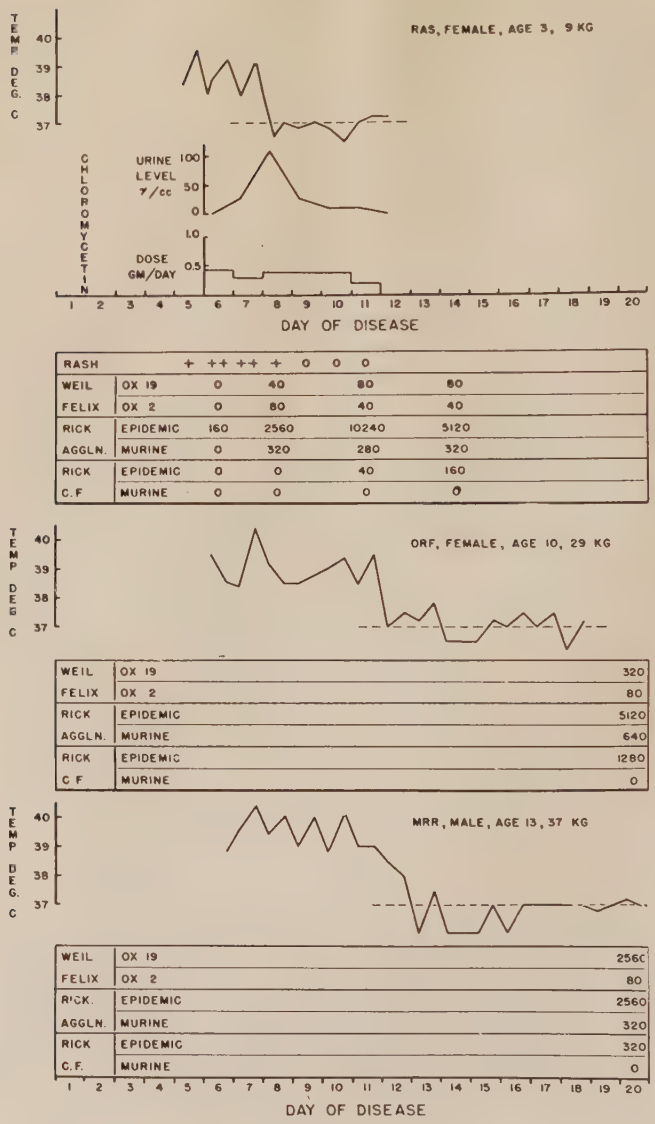


FIG. 6.

whom the prognosis on the eighth day was ominous, who recovered more rapidly than one would have expected.

Fig. 4 summarizes the data on temperature, pulse and serological response of an untreated adult case of epidemic typhus observed in the Hospital General during the period of study.

During the investigation 7 cases of typhus fever were followed in the Hospital del Niño. On the basis of serological findings, 4 of these subsequently were proved to be murine typhus and 3 epidemic typhus. One child with each type of disease was treated with Chloromycetin; the detailed records on these 2 treated children are illustrated in Fig. 5 and 6, respectively. Fig. 5 also contains information on the 3 untreated children who had murine typhus, and Fig. 6 the data on the 2 untreated children with epidemic typhus. In general, the children received orally amounts of drug which were comparable to that given to adult patients on the basis of body weight. Thus, patient P.G.P. received about as much as A.F., the first patient treated,

and patient R.A.S. was given a dosage intermediate between that given to adults A.F. and R.L. Typhus fever in children is generally so mild that evaluation of any therapeutic agent is extremely difficult even when large numbers of cases are investigated. It was our impression that Chloromycetin therapy produced no untoward effects in these children and may have been of some benefit.

Conclusion. On the basis of these limited observations, it may be concluded that the administration of Chloromycetin to patients with typhus fever is a relatively safe procedure. Furthermore, the chemotherapeutic effect obtained in the few patients treated was sufficiently encouraging to warrant further tests with the drug. It is suggested that chloromycetin be employed in oral treatment of the next group of patients to be studied according to the following schedule; an initial dose of 40 mg per kilo body weight followed by a total daily dose of 35 mg per kilo body weight, given in divided amounts at 2-hour intervals, until obvious improvement in the patient's condition is noted; subsequently maintenance dose of 20 mg per kilo body weight per day given in divided amounts at 4-hour intervals, until the 13th or 14th day after onset.

16379

Active and Inactive Murine Poliomyelitis Virus as Interfering Agents Against Poliomyelitic Infection in Monkeys.*

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It has previously been shown that live mouse-adapted human poliomyelitis virus has the ability of interfering with the propagation of simian poliomyelitis virus in rhesus monkeys. Such interference is demonstrable (a) by the intracerebral injection of a mixture of murine (SK, MM) and simian (Aycock, RMV) virus, or (b) by the peripheral administration of large amounts of murine virus during the early incubation period of poliomyelitic infection.[1] The mechanism of this antagonism is not known even though several hypotheses have been advanced as possible explanations.[2,3]

In an attempt to throw more light on this problem it seemed of interest to investigate whether the interfering effect can be elicited with inactivated or attenuated murine virus as well as with active virus. The following report deals with an exploration of this question. The murine virus used was the MM strain, the simian virus was the Aycock strain. The methods chosen for inactivation were (a) desiccation, (b) exposure to radioactive sodium, (c) ultraviolet irradiation, and (d) chlorination.

Methods: Desiccation. Brains from infected mice were harvested at the height of paralysis, placed in an open Petri dish, and dried in a desiccator over sodium hydroxide at 37°C., 20-22°C or 4°C for various lengths of time. The procedure was carried out either *in vacuo* or in nitrogen atmosphere. It was found that the virus preserved its full virulence (10^{-9} to 10^{-10} i.c. or i.p.) for the longest period tested, *i.e.* 20 days, when dried in the icebox at 4°C. At room temperature (20-22°C.) the virus suffered a progressive loss of virulence

* Aided by grants from the Philip Hanson Hiss, Jr., Memorial Fund and from anonymous donors.

1 Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407; Sanders, M., and Jungeblut, C. W., *J. Exp. Med.*, 1942, **75**, 631; Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1942, **76**, 127.

2 Jungeblut, C. W., *J. Exp. Med.*, 1945, **81**, 275.

3 Gard, S., *Acta Med. Scand.*, 1944, **119**, 27.

which began at 5 days and became more marked at 10-15 days; after 29 days drying the remaining potency was such that mice could only be infected intracerebrally with a 10^{-1} dilution. This inactivation, however, could be largely prevented by drying in nitrogen atmosphere. When desiccation was carried out in the incubator (37°C) a rapid loss of virulence occurred so that virus dried for 5 days was barely infectious in a 10^{-1} dilution by intracerebral test; the same dose produced no symptoms after intraperitoneal inoculation. Virus thus attenuated was used for the later experiments.

Exposure to radioactive sodium. A 10% virus suspension, prepared from freshly harvested infected mouse brains, was combined in equal volumes (1 ml) with radioactive sodium (Na^{24}) in isotonic sodium chloride solution at pH 5-6.† The amounts of radio-sodium used varied from 20 to 120 mg NaCl and the radioactivity from 1 to 10 millicurie, as measured with the Geiger-Muller counter at the time of initial contact with the virus. After various periods of contact, ranging from 24 hours to one week in the icebox, the preparations were sampled and viral potency was determined. Within the first 48 hours there was little if any loss of virulence. After 96 hours contact, some of the preparations had deteriorated to the extent that only 10^{-1} dilutions were infectious for mice, while other preparations apparently had retained their full virulence. No further change occurred after longer contact up to one week. Virus titrations could only be carried out by the intraperitoneal route, since intracerebral injections proved toxic for mice. Since the procedure gave extremely irregular results in repeated tests and failed to yield preparations which were non-infectious in a 10^{-1} dilution by intraperitoneal test in mice, none of these preparations were employed in later experiments. One more reason for abandoning these preparations was the fact that virus attenuated by radioactive sodium proved capable of causing a lethal burn in the brain of a monkey following intracerebral injection, even though the mixture, through standing for 4 days, had lost the greater part of its initial radioactivity.

Ultraviolet irradiation. Through the courtesy of Dr. S. O. Levinson of the Michael Reese Research Foundation, Chicago, an opportunity was afforded to test several murine virus preparations which had been inactivated by flash exposure to extreme ultraviolet irradiation with the high power, low pressure lamp used by the Chicago group of workers. The exposure time varied from 0.2 to 0.5 second. Preparations irradiated for 0.3 second proved uniformly non-infectious for mice in 10^{-1} dilution by i.c. or i.p. test.

Chlorination. Solutions of sodium hypochlorite were prepared which contained various amounts of the substance ranging from 42 parts per million to 4200 parts per million. Equal volumes of the NaClO solution and of a non-centrifuged 10% viral mouse brain suspension were combined and samples were tested at different lengths of contact for infectivity in mice. It was found that concentrations of 42, 420, 1250 and 2100 ppm had caused no deterioration of potency. However, concentrations of 3150 ppm regularly inactivated the virus completely after 30-45 minutes contact in the icebox, as determined by i.c. and i.p. tests in mice. Material thus prepared, without neutralization of the residual chlorine, was used for later experiments in monkeys.

Interference experiments in monkeys. A number of experiments were run in which rhesus monkeys, following intracerebral infection with 50-100 minimal paralytic doses of Aycock virus (0.5 ml 1:100 *glycerinated* cord suspension) received daily injections by peripheral routes (intravenous, intraperitoneal) of massive doses (10-15 ml of 10% viral mouse brain suspension) of murine virus inactivated by desiccation, by ultraviolet irradiation or by chlorination. These injections were commenced on the day of infection and maintained during the incubation period of the disease, but in no case exceeding 12 days.

† The sodium isotope used in these experiments was prepared in the Radiation Laboratories of the Physics Department, Columbia University, and was kindly supplied by Dr. Allan F. Reid.

TABLE I.

Comparison of the Efficacy of Active and Inactive MM Murine Virus as Interfering Agents Against Infection with Simian Virus in Monkeys.

Interference test.						Controls					
			Result						Result		
Interfering agent	No. of monkeys	Simian virus (glycerinated cord)	Com. P†	Partial P	No P	Type of control	No. of monkeys	Simian virus (glycerinated cord)	Com. P	Partial P	No P
Active MM virus*	24	0.5 ml Aycock virus 1:100 i.c.	3	5	16						
Inactive MM virus (Desiccation)*	6	,,	5	1	0	Untreated	19	0.5 ml Aycock virus 1:100 i.c.	16	3	0
Inactive MM virus (Ultraviolet irradiation)*	5	,,	4	1	0						
						Untreated	9	,,	9	0	0
Inactive MM virus (Chlorination)*	16	,,	11	0	5	Normal mouse brain	3	,,	3	0	0
						Chlorinated normal mouse brain	5	,,	4	1	0
						NaClO	2	,,	2	0	0

* Dose administered: 10-15 ml 10% viral mouse brain suspension daily for a maximum period of 12 days. † P = Paralysis.

TABLE II.

Chlorinated MM Murine Virus Used as Interfering Agent Against Infection with Simian Virus in Monkeys.

Interference test						Controls					
			Result						Result		
Interfering agent	No. of monkeys	Simian virus (fresh cord)	Com. P†	Partial P	No P	No. of monkeys	Simian virus (fresh cord)		Com. P	Partial P	No P
Inactive MM virus (chlorination)*	8	0.5 ml Aycock virus 1:100 i.c.	8	0	0	1	0.5 ml Aycock virus 1:100	i.c.	1	0	0
						1	0.5 ,, ,, ,, 1:1000	,,	1	0	0
	4	0.5 ml Aycock virus 1:1000 i.c.	4	0	0	1	0.5 ,, ,, ,, 1:2000	,,	1	0	0
						1	0.5 ,, ,, ,, 1:10,000	,,	1	0	0
	4	0.5 ml Aycock virus 1:2000 i.c.	4	0	0	1	0.5 ,, ,, ,, 1:50,000	,,	1	0	0
						1	0.5 ,, ,, ,, 1:100,000	,,	1	0	0

* Dose administered: 10-15 ml 10% viral mouse brain suspension daily for a maximum of 12 days.
† P = Paralysis.

An appropriate number of monkeys, infected intracerebrally with the same dose of Aycock virus, and left either untreated or treated with 10% normal mouse brain suspension, with chlorinated normal mouse brain suspension, or with sodium hypochlorite solution (3150 ppm), accompanied these experiments. To provide a basis for comparing the relative efficacy of the several inactivated virus preparations with that of active virus, a number of monkeys were included which received similar injections of unmodified 10% viral mouse brain suspension following intracerebral infection with the same dose of Aycock virus. The results are brought together in Table I.

It appears from the data given in Table I that monkeys which had received ultraviolet-irradiated or desiccated MM virus developed paralysis in the same manner as did the corresponding control animals. When chlorinated MM virus was used as interfering agent there was some evidence of slight but irregular protection. Thus, of a total of 16 monkeys treated in this manner, 5 animals (31.2%) escaped paralysis, whereas all of 19 control monkeys succumbed to the disease. By contrast it will be seen that in a group of 24 monkeys which had received unmodified murine virus, 16 animals (66.6%) remained free from paralysis as compared with a morbidity rate of 100% in a control group of 19 untreated monkeys. The latter figures are in close agreement with the experience previously collected in similar experiments.

In order to determine whether the results obtained with chlorinated murine virus could be duplicated in monkeys infected with a larger dose of simian virus, the experiment was repeated with another group of monkeys. The interfering agent was again MM virus, inactivated by chlorination (2600-3500 ppm sodium hypochlorite), but the infecting dose of Aycock virus ranged from at least 50-1000 minimal paralytic doses (0.5 ml of 1:100 to 1:2000 *fresh* monkey cord suspension). An adequate number of untreated control monkeys accompanied this experiment. The results are shown in Table II.

It will be seen from Table II that all of the treated monkeys developed the typical disease as did the corresponding control animals.

Conclusions. The sum total of the work reported leads to the conclusion that MM virus, inactivated by exposure to various physical and chemical agencies, is unable, as a rule, to prevent the development of paralysis in monkeys infected with simian virus under the conditions employed in these tests. The slight and irregular protection observed on occasion with the use of chlorinated murine virus is exceptional and operates only against limited doses of simian virus. The results are, therefore, in marked contrast with the high rate of non-paralytic survival that is obtainable when unmodified active virus is used as interfering agent in comparable amounts. While interference with active murine virus still offers the only successful approach—known at present—to a prevention of the experimental disease, the fact that inactivation of the virus causes a definite qualitative or quantitative alteration of the effective interfering factor opposes serious difficulties to a possible application of this method for human use.

16380

Studies on 3, 4-Dimethyl-5-Sulfanilamido-Isoxazole (Nu-445) in Humans.*

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Sarnoff, Freedman and Hyman[1] reported on the activity of a new sulfonamide, 3,4-dimethyl-5-sulfanilamido-isoxazole (Nu-445)‡ in urinary tract infections with *B. proteus*. This sulfonamide derivative was found to be characterized by low toxicity and by a high solubility at pH 6.0 to 7.5,[2] the latter greatly diminishing the danger of crystallization in the urinary tract. The compound seemed suitable for the treatment of urinary infections because of its *in vitro* activity against the gram-negative organisms frequently occurring in urinary tract infections.[2]

Our encouraging early studies were subsequently confirmed by Haines and Micelli,[3] Narins,[4] and Lazarus and Schwarz.[5] A less optimistic report has been published by Rodgers and Colby.[6]

The clinical therapeutic work described above[1] was preceded by pharmacological studies in humans. These were mainly concerned with the absorption and elimination of the drug after oral and parenteral administration and the toxicology of the new product in human beings.

The results are presented in this paper.

Determination in blood and urine. Nu-445 may be determined by the method of Bratton and Marshall.[7] A solution of the lithium salt of Nu-445 served as the standard.

Mode of administration. Like other sulfonamides, the oral tablet is 0.5 g. Parenteral administration is facilitated to a considerable degree by the use of the lithium salt of Nu-445. Made up as a 10% solution, each ampule contains 10 cc, or 1 g, and may be injected intravenously or intramuscularly without untoward effects.

Intravenous administration. Three patients of varying weights were given 4 g (40 cc of the 10% solution) of the lithium salt of Nu-445 intravenously. The injection was completed in 3 minutes. There were no untoward reactions. Blood and urine levels were determined at regular intervals during the 24-hour period following the injection. The results of the blood level determinations are given in Table I. Renal excretion of the drug was in evidence 30 minutes after the injection, and the average level in the urine for the ensuing 24-hour period was 500 mg %. Excretion was not complete 24 hours after the injection at which time the determinations were discontinued. The figure of 500 mg % refers to total drug, 35% of which was present in the acetylated form. This is in agreement with the laboratory data of Schnitzer, *et al.*[2] who found 32% of the excreted sulfonamide to be in the acetylated form.

Intramuscular administration. Three g (30 cc of a 10% solution) of the lithium salt of Nu-445 were administered intramuscularly, 15 cc into each gluteal region. The data in Table I indicate that the concentration in the blood was somewhat lower than after the higher intravenous dose. What is ordinarily thought to be a therapeutic sulfonamide

* This study was aided by a grant from the Elsa and William Menke Fund.

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‡ Supplies of this compound were obtained for study from Hoffmann-La Roche, Nutley, N.J.

1 Sarnoff, S. J., Freedman, M. A., and Hyman, A. A., *J. Urol.*, 1946, **55**, 417.

2 Schnitzer, R. J., Foster, R. H. K., Ercoli, N., Soo-Hoo, G., Mangieri, C. N., and Roe, M. D., *J. Pharm.*, 1946, **88**, 47.

3 Haines, W. H., and Micelli, S., *Pennsylvania Med. J.*, 1947, **50**, 1328.

4 Narins, L., *J. Urol.*, 1948, **59**, 92.

5 Lazarus, J. A., and Schwarz, L. H., in press.

6 Rodgers, R. S., and Colby, F. H., *J. Urol.*, 1948, **59**, 659.

7 Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

TABLE I.
Blood Level of Nu-445 After Single Doses Administered by the Oral, Intramuscular, and Intravenous Route.

Dose, g	Route	No. of patients		Avg blood level (mg %) ½ hr	2-3 hr	4-5 hr	8-9 hr	24-30 hr
3	*per os*	3	free	5.8	13.8	16.0	11.0	—
			total	6.5	15.3	17.0	12.7	—
4	" "	3	free	6.8	9.0	14.6	9.2	3.8
			total	7.5	12.0	19.6	16.0	3.8
3	intramuse.	1	free	10.0	15.0	13.0	10.0	3.0
			total	10.0	16.0	16.0	14.0	6.0
4	intraven.	3	free	38.0	26.0	18.6	9.0	2.0
			total	38.0	26.0	20.0	14.0	3.3

level was, however, maintained for an 8- to 9-hour period, and even after 24 hours appreciable blood concentrations were still present. The acetylation varied from 0 to 50%. The average concentration in the urine was 150 mg %, 46.5% of which was present in the acetylated form. Excretion was not complete after 31 hours at which time observations were terminated.

Oral administration. Single oral doses of 3.0 and 4.0 g each were given to 2 groups of 3 patients. In the first group (3.0-g dose), food had been withheld for 12 hours, while the other group received the drug coincident with the noonday meal. The results of the blood levels as given in Table I do not seem to indicate that the presence of food in the stomach greatly alters the absorption. Adequate blood concentrations were maintained for a period of 2 to 9 hours. Urine determinations were carried out on specimens obtained 4 and 8 hours after the ingestion of 3 g *per os* in 3 patients. An average value of 236.6 mg % of total drug was found, 32.8% of which was in the acetylated form.

TABLE II.
Variation in Blood Level Over the 8-Hour Period. Patient Has Been Receiving 2 Grams Nu-445 *per os* Every 8 Hours for 24 Hours.

Nu-445 *per os*	Time	Patient A Free mg %	Patient A Total mg %	Patient B Free mg %	Patient B Total mg %
	12	13	17	13	18
2 g	2	10.5	14	10	16
	4	20	22	16	20
	6	16.5	20	15	19
	8	12	14	11.5	16
2 g	10	10	14	11	15

Multiple oral doses were first tried by adopting a dosage schedule of 2.0 g every 8 hours. This would confer several advantages in that it would halve the number of medications by the nursing personnel, and allow an 8-hour period during which the patient's sleep would not be disturbed. Table II indicates that such a dosage schedule does not allow the blood level to fall below the desired concentration at any time during the 8-hour period.

It seemed desirable to study dosage schedules in which a high initial dose was followed by the continued administration of smaller doses over a longer period. The blood levels observed in patients while on 3 different dosage schedules are given in Table III. Blood samples were taken midway between doses. The figures show that a consistent level was maintained for the entire period of medication. Twenty-four hours after discontinuation of the drug, the concentration of the drug in the blood was found to be quite low. Urine samples taken during the period of treatment showed an average concentration of 425.5 mg %, 34.8% of which was present in the acetylated form. Twenty-four hours after the administration of the drug was stopped, the urine concentration dropped to 12-18 mg % total drug, 59% of which was acetylated. Traces of diazotizable material could still be demonstrated in the urine of a patient 3 days after a total dose of 46.0 g, over a 7-day period, had been given.

Spinal fluid. Since the activity of Nu-445 against the meningococcus was found to be similar to that of sulfadiazine,[2] the rapidity of its entry into the spinal fluid and the

TABLE III.
Blood Level of Nu-445 After Different Oral Dosage Schedules.

Dosage	No. of patients		Avg blood level in mg % after days 1	2	3	4	5	6	7	8
4 g initial	1	free	12.0	12.5	11.0	10.0	10.0	11.0	11.0	1.5*
1 g every 4 hr		total	12.0	18.0	16.0	14.0	14.0	18.0	16.0	1.5*
3 g initial	3	free	16.0	12.3	15.0	13.8	15.8	1.7*	—	—
2 g every 8 hr		total	19.3	17.0	18.0	18.0	19.0	2.5*	—	—
3 g initial	2	free	10.7	11.0	12.3	13.0	12.0	—	—	—
1 g every 6 hr		total	16.7	16.0	17.5	17.0	17.0	—	—	—

* Medication discontinued the night before.

eventual level it attains there are of some importance. Table IV indicates that, within 2 to 3 hours after its intravenous or oral administration, the drug begins to enter the spinal fluid. In 2 patients with normal central nervous systems the eventual levels attained were low, being 1.2 mg and 1.0 mg % while blood levels were 7.5 and 11.5 mg %, respectively, of the free drug. However, in one patient with meningococcus meningitis the intravenous injections of 4 g and continued oral doses resulted in blood levels of between 28-30 mg % free and 37-40 mg % of the total drug. There was clinical and hematologic evidence of severe dehydration in this patient. At this time the spinal fluid levels were 8 mg % free and 36 mg % total Nu-445. A subsequent spinal fluid level determination revealed 5 mg % free and 12 mg % total drug. Recent evidence indicates that the barrier between blood and spinal fluid is appreciably altered in the presence of inflammation.

Toxicity in the human. Nu-445 was administered in varying doses to 37 patients. Other than the 6 patients who were given single doses at various times for the purposes of absorption studies, this group received total doses ranging from 17 to 93 g over periods ranging from 4 to 20 days. No alkali therapy was given. No attempt was made to control the hydration of the patient in order to duplicate the various conditions which one might expect to encounter clinically.

One significant fall in hemoglobin, 22%, was encountered in a patient with ulcerative colitis and a 4+ guaiaic test in the stool during the entire course of drug administration. One moderate depression of the white blood cell count occurred in a severely debilitated individual with pyelonephritis in whom a provisional diagnosis of Hodgkin's disease had been made. One patient experienced a 2° rise in temperature coincident with the onset of drug administration. He was in his 6th postoperative day, but no evidence of infection was present and the fever was attributed to the drug.

TABLE IV.
Early Spinal Fluid Levels After Oral and Parenteral Administration of Nu-445.

Dose	Hr after administration	Mg % Free	Total
4 g. p.o.	2.5	0.25	0.25
4 "	3.5	0.025	0.025
4 "	2.8	0	0.15
4 g i.v.	2.0	0.4	0.8
4 "	2.6	0.5	0.75

One patient had reacted with severe urticaria and vomiting to as little as one g of sulfadiazine on 3 different occasions, but did not react to full doses of Nu-445 over a period of 2 weeks. A second patient had experienced severe headache, nausea, and vomiting with the previous administration of either sulfadiazine or sulfathiazole. The first dose of 4 g of Nu-445 was followed by a moderate headache, but this disappeared in 24 hours and no further reaction was noted while the patient was on full doses.

Crystalluria was not observed in any patient despite the fact that no alkali was given. Hematuria attributable to the drug was not

observed, nor was there any evidence of renal irritation.

Thirty cc of a 10% solution of the lithium salt were injected intramuscularly (15 cc into each buttock) in 3 patients. The injection was tolerated well in each case and no subsequent discomfort was noted.

One patient, not included in the above group, afforded the opportunity for massive oral and intravenous therapy. Subacute bacterial endocarditis with *Bacillus proteus* had been present for several months. In view of the activity of Nu-445 against this organism in the genito-urinary tract[1] and *in vitro,* it was thought worthwhile to attempt massive therapy in this case. After a 4-g initial dose, 1 g every 4 hours was administered for 4 days, 2 g every 4 hours for 4 more days, and then 3 g every 4 hours for 10 days. No reactions were noted. While the patient was receiving this oral dose, 18 g of the lithium salt of Nu-445 were given intravenously over a period of 4 hours. Two days later, with the patient still on the same oral dose, 12 g were given intravenously in 10 minutes. Pallor and sweating were noted for a few hours after this last dose and crystalluria and microscopic hematuria were noted for the ensuing 3 days. This was accompanied by a transient rise in the blood urea nitrogen. It is doubtful if the usual clinical requirements would ever indicate the administration of even a fraction of the dose administered to this patient.

Two other patients not included in the above group who had been hospitalized because of recent coronary occlusions, had fatal recurrences while receiving drug therapy. There was post-mortem confirmation in one of these. A third patient, recovering from a recent, severe, postoperative pulmonary embolization was being treated with Nu-445 in an attempt to clear his urinary tract of a *Bacillus proteus* infection, and while under treatment sustained a fatal recurrence.

Summary. A new sulfonamide preparation has been presented which, on the basis of its high solubility in buffer solution, would be expected to greatly diminish the danger of sulfonamide crystallization in the kidney, with or without the concomitant administration of alkali. Neither crystallization nor the signs of renal irritation in the absence of crystallization were observed (except in the patient to whom enormous doses were administered). The antibacterial activity *in vivo* and *in vitro* and the low toxicity in the experimental animal combined to recommend an investigation of the clinical pharmacology of this compound. The blood and urine levels attained were found to be satisfactory and it was found possible to achieve and maintain these levels with an 8-hour dosage schedule. The entry of this drug into the spinal fluid of the normal individual is low, but in the presence of meningeal inflammation it was markedly increased in the one patient studied. The toxicity of the compound is low.

16381

Action of Quaternary Ammonium Salts and the Theory of Competitive Inhibition of Acetylcholine.

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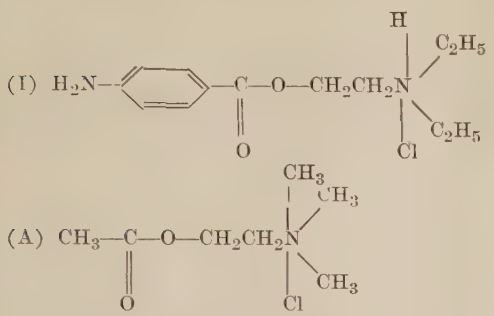
In several publications[1,2,3] attempts have been made to explain local anesthesia as a competitive inhibition of acetylcholine action upon the nerve. The theory, of course, assumes acceptance of the acetylcholine theory for the transmission of impulses[4] along the nerve fibre and the nerve ends. Some assume that similarity in chemical structure enables

a local anesthetic like procaine hydrochloride (I) to substitute for acetylcholine chloride (A) in the nerve tissue:



However, it is hazardous to ignore the extreme differences in physiological and chemical properties between quaternary ammonium salts and salts of tertiary amines. A study of quaternary ammonium derivatives closely related to acetylcholine chloride and to procaine hydrochloride should shed some light on the problem.

If the theory cited is to be accepted, the nerve "receptor" cells must be assumed to be unable to distinguish between a tertiary amine salt and a quaternary ammonium salt; quaternary ammonium salts of the local anesthetic esters should either antagonize or potentiate the acetylcholine effect.

The compounds used in this study were procaine hydrochloride (I) and the following closely related products, in which only the C_2H_5, H or Cl groups attached to the terminal N of I were varied:

I. C_2H_5, C_2H_5, H, Cl Procaine hydrochloride
II. C_2H_5, C_2H_5, C_2H_5, I Procaine ethiodide
III. C_2H_5, C_2H_5, CH_3, I Procaine methiodide
IV. CH_3, CH_3, H, Cl
V. CH_3, CH_3, CH_3, I *p*-Aminobenzoylcholine iodide
VI. CH_3, CH_3, C_2H_5, I

Compounds I and IV are hydrochlorides of tertiary amines, the rest quaternary ammonium derivatives. I, II, and IV, were first prepared by Einhorn.[5] Compound III was furnished by Mr. F. N. Minard. Compounds V and VI which appear to be new, were characterized as follows:

V. β-dimethylaminoethyl *p*-aminobenzoate methiodide. m.p. 254-5°. Anal. calcd. for $C_{12}H_{19}N_2O_2I$: N, 8.00. Found: N, 8.08.
VI. β-dimethylaminoethyl *p*-aminobenzoate ethiodide. m.p. 113-6°. Anal. calcd. for $C_{13}H_{21}N_2O_2I$: N, 7.69. Found: N, 7.58.

Rapp and Wessinger[2] had described a marked diminution of the depressor effect of acetylcholine by a preceding (presumably very large) dose of procaine. Their paper refers to only one experiment in a dog and does not state the amount of procaine given. Hence it seemed desirable to repeat their experiment. In our tests 5 dogs weighing 7-10 kg and 2 cats of 3.2 kg and 2.5 kg were used. Four dogs and 2 cats were under Pentobarbital (Nembutal) anesthesia; one dog had been decerebrated under ether. Acetylcholine, 1-5 γ, was injected repeatedly i.v. in order to cause reproducible blood pressure drops of 30-50 mm Hg. After this effect had been established, procaine hydrochloride was given i.v. and followed immediately by repeated acetylcholine injections. No change in response to acetylcholine was seen in the cats following the administration of 10-20 mg procaine hydrochloride. The same result was seen in the 4 anesthetized dogs when 40-50 mg of procaine hydrochloride were used; however, 100 mg in one of the dogs caused a reduction of response to acetylcholine by about 30%. In the decerebrated animal 40-60 mg of procaine hydrochloride inhibited acetylcholine action by 30-50% respectively. (See Fig. 1, part I) In only one of the dogs (6 kg) under nembutal anesthesia did we succeed in obtaining a significant decrease of the typical depressor effect of acetylcholine by smaller doses of procaine (4-10 mg) during one part of the experiment. We were unable to detect the circumstances which caused or prevented this responsiveness; anesthesia and height of blood pressure were not involved. Procaine hydrochloride itself in doses below 100 mg did not cause a visible effect upon the blood

[1] Thimann, K. V., *Arch. Biochem.*, 1943, **2**, 87.

[2] Rapp, G. W., and Wessinger, G. D., *The Burr.*, 1944, **44**, 58.

[3] Schueler, F. W., *J. Chem. Education*, 1945, **22**, 585.

[4] Nachmansohn, D., in Physical-Chem. Mechanism of Nerve Activity, *Ann. Acad. Science*, 1947, **47**, 395.

[5] Einhorn, A., *Ann.*, 1909, **371**, 125.

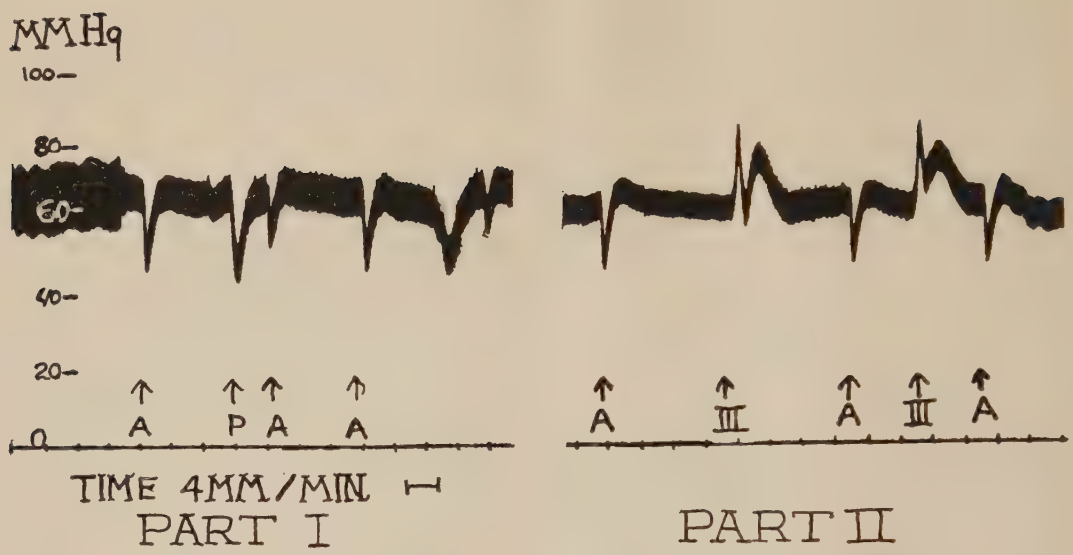


FIG. 1.
Dog, 6 kilo, decerebrated blood pressure taken from the carotid artery. A = 5 gamma acetylcholine. P = 40 mg procaine HCl. III = 4 mg cpd. No. III.

pressure in dogs. At 100 mg it produced a slight and transitory depression. If any effect upon the response to acetylcholine occurred, it lasted but 3 minutes.

Similar experiments were carried out with compound IV. When this dimethyl analogue of procaine was injected in doses of 50 mg in a cat weighing 2.5 kg, no reduction or an only insignificant one, in the typical response of blood pressure to acetylcholine occurred.

Acetylcholine is known to cause a marked contraction of the isolated rabbit intestine *in vitro* by peripheral parasympathicotropic stimulation. When 1.0 mg procaine hydrochloride was added to a bath containing 20 cc of Tyrode solution, a moderate reduction in tone and amplitude of contractions occurred, but the response to 20 γ acetylcholine remained unimpaired.

Richards, Roth and Kueter[6] have reported that compound II possesses definite nicotine-like properties and produces a marked pressor effect frequently followed by a short depressor phase in dogs; in cats the depressor action prevailed. Further work, which will be described elsewhere in detail, indicates that compounds III, V, and VI have a similar pressor effect in both species. These substances are completely devoid of local anesthetic activity, but have curare-like properties.

The action of acetylcholine upon blood pressure was studied in 2 anesthetized cats and 3 anesthetized and one decerebrated dog before and following single or repeated doses of 1-4 mg of Compounds II, III, and V, immediately after the blood pressure had returned to the base line and then again about 1-2 minutes later. In no case did we notice a change of the typical acetylcholine effect. Fig. 1, part II shows a typical experiment using Compound III. The same results were obtained with Compounds II, V, and VI.

In experiments on isolated rabbit intestine, analogous to those described above, Compound II did not exert an effect in doses of 1.0 mg, Compound V produced a transitory stimulation, and neither affected the typical response of the gut to acetylcholine.

Discussion. Recent publications such as the ones by Gerard[7] and Heymans,[8] have

[6] Richards, R. K., Roth, L. W., and Kueter, K. E., *Fed. Proc.*, 1947, **6**, 364.

[7] Gerard, R. W., in Physical-Chemical Mechanism of Nerve Activity, *Ann. New York Acad. Science*, 1947, **47**, 575.

[8] Heymans, G., and Jacob, F., *Arch. Intern. Pharm.*, 1947, **74**, 233.

cast serious doubt upon a role of acetylcholine and its esterase in the mediation of conduction along the nerve fiber. Nevertheless, the importance of this substance in the mediation of the stimulus at nerve endings cannot be completely excluded. In our experiments we were not uniformly able to repeat the inhibition of the depressor action of acetylcholine by procaine as reported by Rapp and Wessinger, but it appeared that under the influence of relatively large doses of procaine a moderate decrease of the acetylcholine effect could be obtained. Our results on the isolated rabbit intestine were completely negative in this respect. We doubt that the above data can properly be interpreted as supporting the theory of a competitive inhibition of acetylcholine by procaine on the nerve ends.

As pointed out, procaine is the hydrochloride of a tertiary amine and cannot justly be considered a quaternary ammonium compound; II, III, V, and VI are true quaternary ammonium derivatives, and are therefore chemically more like acetylcholine than procaine; this is particularly true of compound V which contains 3-methyl substituents as does acetylcholine. None of the quaternary derivatives possessed the effect typical of acetylcholine, nor did they interfere with its action upon blood pressure or isolated rabbit intestine. The compounds exerted, in many respects, rather a marked nicotine-like effect.

If partial inhibition of acetylcholine action by procaine is to be ascribed to similarity in chemical structure, such inhibitory effects should logically be expected to increase in the case of the closely related quaternary ammonium derivatives. This was not observed.

Summary. The methyl and ethyl quaternary ammonium salts of procaine and of the analogous compound in which the ethyl groups are replaced by methyl groups were prepared. They were studied pharmacologically, especially with regard to their ability to modify some of the effects of acetylcholine, and their properties were compared with those of the hydrochlorides of the tertiary amines. No support was found for the theory that inhibition of acetylcholine action in this series is dependent upon similarity in chemical structure.

16382

Fate and Distribution of Penicillin in the Body. I. Circulation of Penicillin in the Lymph.*

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The presence of penicillin in the urine[1,2] and in the tissues[3,4] long after its disappearance from the blood stream, posed a number of questions concerning the fate and distribution of the antibiotic in the body. This led us to study the penicillin content of the lymph and its relation to the blood levels.

We expected to find an explanation for the missing correlation between blood, urine, and tissue levels in the lymphatic circulation.

Material and Methods. Dogs of various breeds, of 9-12 kg body weight, were anesthetized with either nembutal (35 mg/kg,

* Part of this material was presented at the Meeting of the American Societies of Experimental Biology, Atlantic City, May, 1948.

1 Romansky, M. J. and Rittman, G. E. *New England J. Med.*, 1945, **233**, 577.

2 Cohn, A., Kornblith, B. A., Gruenstein, I., Thomson, K. J. and Freund, J. PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 145.

3 Ercoli, N., Hueper, W. C., Landis, L., Lewis, M. N. and Schwartz, B. S. *Am. J. Med. Sci.*, 1948, **215**.

4 Schwartz, B. S., Lewis, M. N., Whitehead, M. and Ercoli, N., in press.

TABLE I.
Penicillin Content of the Lymph and Serum in Dogs Treated with 20,000 u. Penicillin in Saline.

Dog No.	Route	Hr	Units/cc Serum	Units/cc Lymph
1	Intramuscular	2¼	.5	2.0
		4-5	0	.5
		7	0	2.0
11		6	.03	.125
		7	0	.03
17		7	.03	.06
		8	.03	0
23		3½	.06	.125
		4	0	.06
		5	0	.06
10	Intravenous	3	.125	.25
		4	0	.125
		5	0	.06
		6	0	.03
14		1	2.0	4.0
		2	1.0	2.0

i.v.) or sodium phenobarbital (120 to 160 mg/kg, i.p.). A glass cannula was inserted and tied into the thoracic duct at its entrance into the vein and the lymph was permitted to flow continuously. Samples of blood (femoral vein) and lymph (cannula) were taken periodically and assayed for penicillin. To rule out the possible inherent bacteriostatic properties of these fluids, samples collected before treatment were used as control. For

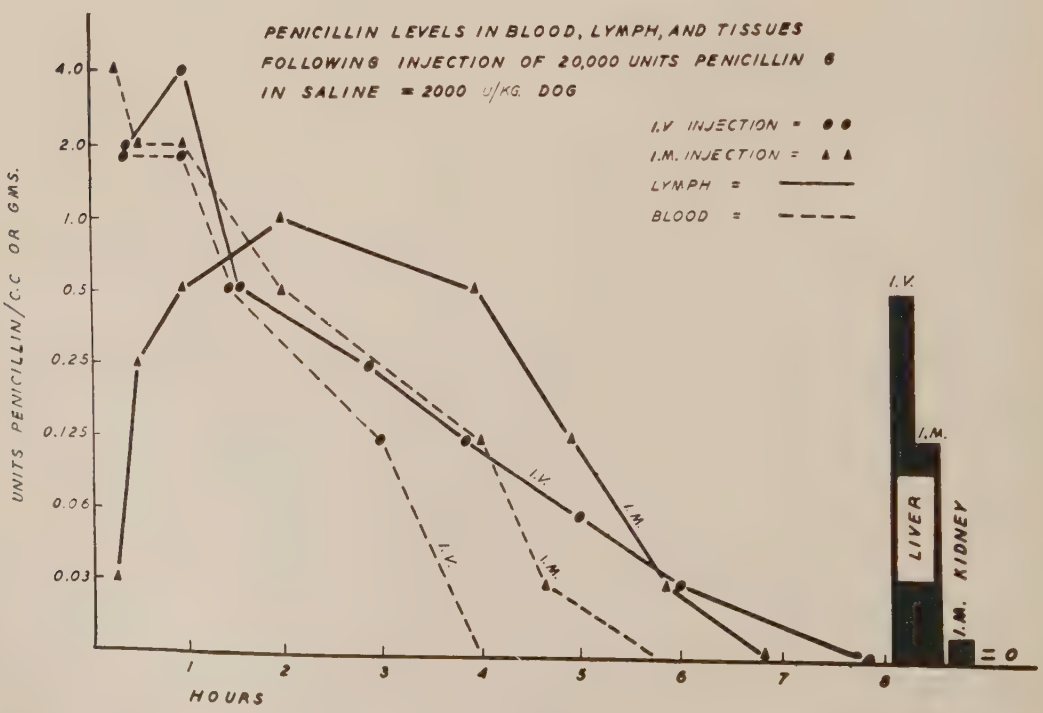

FIG. 1.

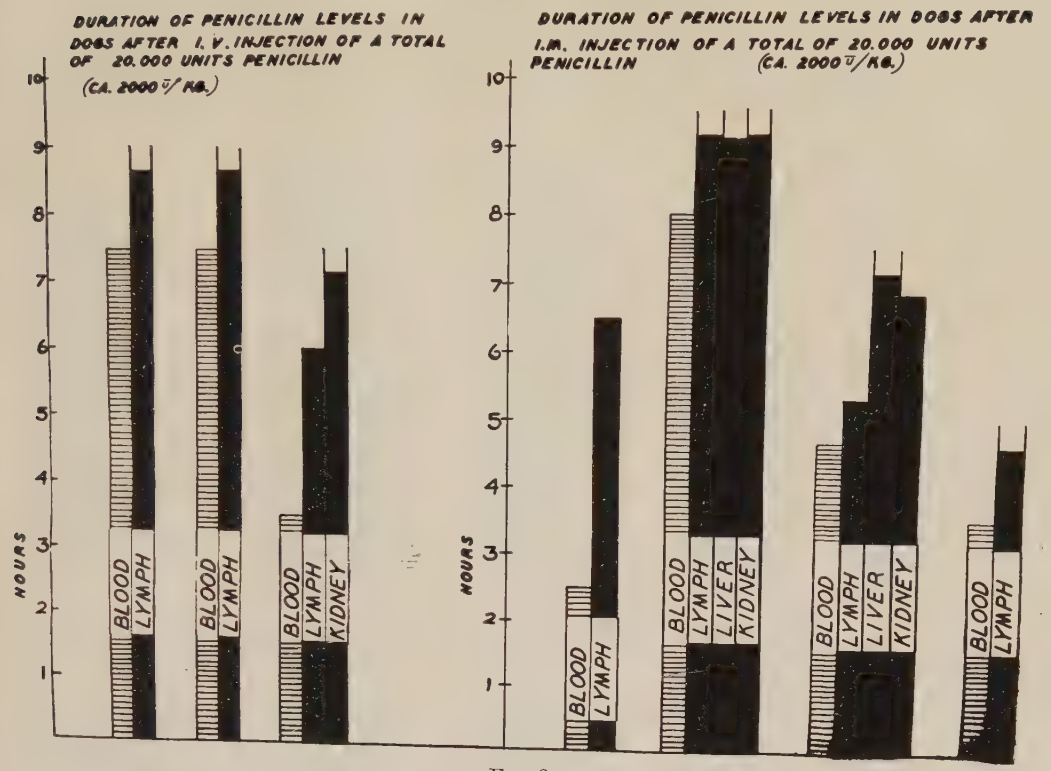

FIG. 2.

penicillin determination, the dilution method,[5] with defibrinated rabbit blood as indicator of hemolysis and β hemolytic streptococcus strain 4 (Group A, Type 3), received in 1946 from Dr. R. J. Schnitzer, was used as test organism. With his method penicillin concentrations of 0.015-0.03 u/cc, are detectable. The tissues collected at the end of the experiment were mechanically ground, taken up in 2 volumes of phosphate buffer (pH 6) and assayed for penicillin. Crystalline potassium penicillin G was administered in saline by various routes and by repository injection. For the latter purpose, we injected (intramuscularly) a suspension of crystalline penicillin in oil plus epinephrine, which gives a high prolongation of blood and organ levels.[3] (A commercial preparation—"Intracillin"—containing 300,000 u penicillin and 0.3 mg epinephrine per cc oil, was used.)

1. *Penicillin in Aqueous Medium.* (a) *Parenteral administration.* A total of 23 dogs were anesthetized and prepared for collection of blood and lymph samples. Table I shows the results of experiments in dogs treated with 20,000 units of penicillin, either intramuscularly or intravenously (approximately 2000 u/kg). By both methods of administration the retention of penicillin in the lymph was longer than in the blood. The lymph and blood level curves did not show the same general direction. One and one half hours after injection, the penicillin content of the blood was rapidly descending; this was not the case in the lymph, as appears in Fig. 1. The duration of detectable penicillin levels in the blood, lymph, and organs is represented in Fig. 2. (b) *Intraduodenal administration.* After laparotomy, the aqueous solution was injected into the duodenum with a hypodermic needle. A 9.5 kg dog, treated with 1,000,000 units, showed a high penicillin blood concentration (0.5 u/cc) within 5 minutes after administration; in ½ hour the concentration increased to 4.0 u/cc. After 8

[5] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

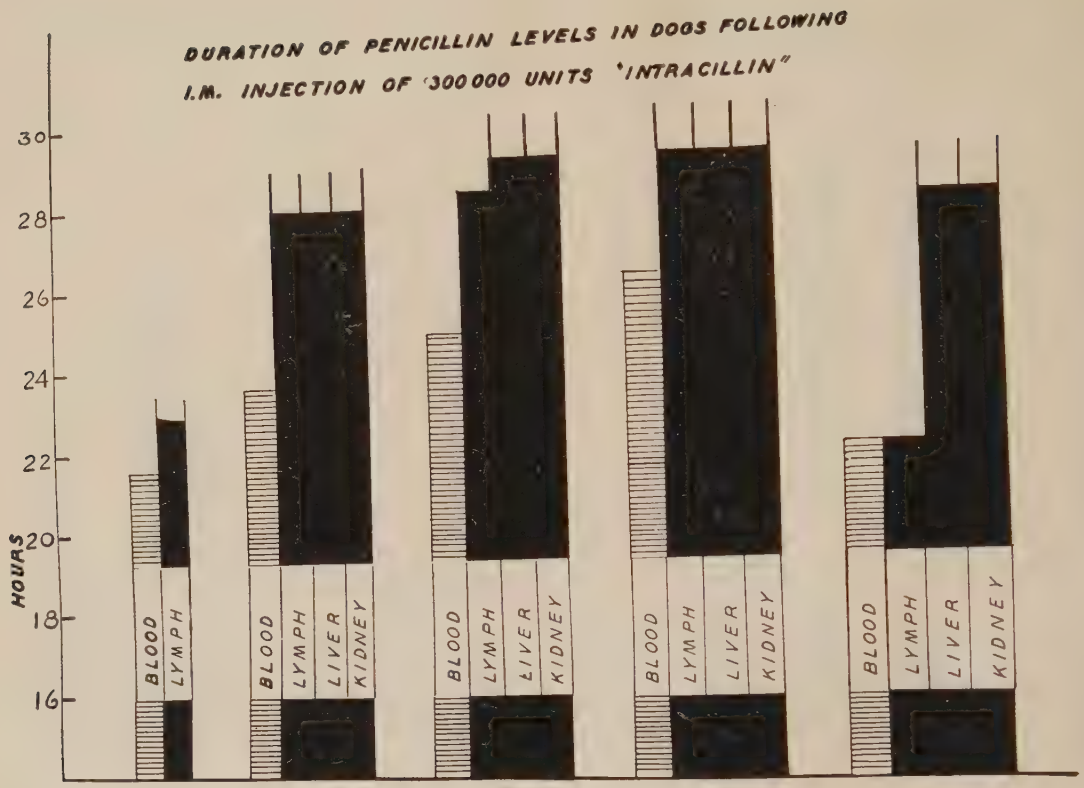

FIG. 3.

hours the blood still contained 0.25 u/cc penicillin. The penicillin did not appear in the lymph until 10 minutes after injection; after this period the lymph level followed the blood level for the entire duration of the observation (8 hours). In a second dog, weighing 9.0 kg, treated with 20,000 u penicillin, 0.5 u/cc was found in the lymph and in the serum during an observation period of 3 hours. A third dog, weighing 12.5 kg treated with the same dose, showed penicillin in the lymph for the complete observation period of 7 hours, whereas the penicillin remained in the blood for only 6 hours. In this experiment the penicillin appeared in the lymph more than one hour after administration, when the blood already contained high penicillin concentrations (0.5 u/cc).

2. *Repository Treatment.* Fifteen dogs were injected intramuscularly with 1 to 3 cc Intracillin, corresponding to 300,000 to 900,000 units crystalline penicillin G. In one group of experiments, the lymph and blood levels were followed immediately after injection; in another group, the dogs were anesthetized for cannulation of the thoracic duct 16 hours after penicillin treatment. During most of the period when penicillin was still detectable in the blood stream, the concentrations in the blood and lymph were of approximately the same value. However, toward the end period of the blood level curve, the lymph concentration was higher in the majority of cases. In relation to this, the duration of the penicillin in the lymph was significantly longer than in the blood. The duration of the detectable penicillin levels in the blood, lymph, liver, and kidney is presented in Fig. 3. The difference in duration between blood and lymph was probably greater than it would appear from these figures, since in most of these long-lasting experiments the lymph level was not followed to the end.

3. *The Time of Appearance of Penicillin in the Lymph.* In a certain number of experiments, the time of appearance of penicillin in

TABLE II.
Time of Appearance of Penicillin in the Lymph and in the Serum.

Minutes after intramuse. inject.		Saline, 20,000	Procaine penicill., 300,000 u	Intracillin, 300,000 u.
5	Lymph	0	0	0
	Serum	0.5	1.0	8.0
10	Lymph	0.25	0.125	0.125
	Serum	2.0	2.0	8.0
15-25	Lymph	—	2.0	2.0
	Serum	—	2.0	8.0

TABLE III.
Organ, Lymph, and Blood Levels of Dogs Treated with Penicillin.

Administration	Dosage, × 10,000	Assay at end of exper. hr	u/cc			u/g	
			Blood	Lymph	Urine	Liver	Kidney
Saline i.m.	2	6½	.25	.03		.25	.5
,, ,,		9	.5	.25		.5	.12
,, i.v.	1	4½	.25	.25	8<	.25	1.0
Intracillin, i.m.	30	23	.25	.25		.5	4.0
		29	0	.5	10	.12*	.5
		30	0	.015		.5	0
	90	36	.06	.12		.125	.125

* Lung and spleen = 0.12 u/g.

the blood and lymph was noted. In every case studied—as in the previously mentioned intraduodenal experiments—the penicillin appeared first in the blood, then, 5 to 10 minutes later, in the lymph. (Table II). These results suggest that the absorption of penicillin takes place through the blood capillaries. It is noteworthy that the recently described insoluble procaine penicillin suspended in oil[6] gave the same result as the soluble salts.

4. *Lymph and Organ Levels.* The duration of penicillin in the liver and kidneys was generally longer than in the lymph. Occasionally it was observed that the duration of the penicillin was more prolonged in the liver than in the kidneys. (Figs. 2 and 3.)

Conclusions. This study reveals that the penicillin lymph level is more prolonged than the blood level. The appearance of penicillin in the blood earlier than in the lymph suggests that the absorption of penicillin takes place through the blood capillaries. It seems to be a regular occurrence that penicillin, after its passage from the blood into the lymphatic system and into the tissues, is retained longer in the last two.

That there is a definite accumulation of penicillin in the organs, particularly following repository injection, is clearly demonstrated by our data on dogs and further substantiated by other rat experiments.[4] The elimination through the lymphatics of this penicillin accumulated in the organs might be responsible for the prolongation of the lymph levels.

The penicillin found in the thoracic duct reaches the blood stream where, following its greater dilution, it becomes bacteriologically undetectable. In all probability, this undetectable penicillin is concentrated in the kidneys and then reappears in high concentrations in the urine.

By analogy, we may assume, from the frequent presence of penicillin in the organs (liver, kidney) after the end of the detectable lymph level, that the penicillin circulation in the lymphatic system is probably longer than can be demonstrated by the methods used.

The therapeutic interest of the prolonged penicillin lymph level is obvious considering that the total volumes of the lymph and of

[6] Hobby, G. L., Brown, E., and Patelski, R. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 6.

the blood are of the same magnitude,[7] and that the "lymph comes closer to reflecting the actual environment of the body cells than any other fluid that can be collected" (Drinker and Joffey[8]).

The well known role played by the lymphatic system in certain infections adds further interest to this observation.

[7] Abel, J. J., Hampil, B., Jonas, A. F. and Chalian, W., *Bull. Johns Hopkins Hosp.*, 1938, **62**, 522.

[8] Drinker, C. K., and Joffey, J. M. Lymphatics, lymph and lymphoid tissue. *Harvard University Press*, Cambridge, Mass. 1941.

16383

Influence of Sodium Tetrathionate on Rate of Blood Flow to the Digits.

TRAVIS WINSOR.*

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Sodium tetrathionate (Tetrathione) has been used extensively by Theis and Freeland,[1-4] primarily for peripheral vascular disorders characterized by deficient oxygenation of the blood and tissues. Most prominent among these are Buerger's disease.[5] Theis and Freeland[1] show, during the active stages of thromboangiitis obliterans, either a decrease in the oxygen capacity or in the degree of oxygen saturation of the arterial blood, as well as increased sedimentation rates without significant departures from the normal in erythrocyte counts and hemoglobin percentages. In these cases venous blood taken from the involved extremities was abnormally dark, showed an increase in viscosity and sedimentation rate, rapid coagulation without an increase in fibrinogen, increased alkalinity and a low degree of oxygen saturation with a low carbon dioxide content. A subnormal content of the oxidation catalyst, the reduced form of glutathione, was noted in the venous blood. Sodium tetrathionate administered intravenously was of value in restoring to normal these abnormal blood findings. Since this work, some controversy over the value of sodium tetrathionate in the treatment of peripheral vascular disorders has arisen.

The present studies were carried out to determine whether or not sodium tetrathionate influenced the rate of blood flow, skin temperature, or pulse rate in normal individuals and in patients with peripheral vascular disease.

Methods and Materials. The rate of blood flow was determined, using a digital pneumoplethysmograph.[6] The occluding cuff was placed at the ankle or wrist. The venous occlusion was accomplished by inflating a cuff with approximately 60 mm of mercury pressure. The rate of volume change was measured by taking the angle between the base line before and after venous occlusion. The volume of the part was measured using the volumometer, as described by Burch.[6] The rate of flow was then determined after a period of rest, not less than 45 minutes. Tracings were taken after the injection of 10 cc of distilled water intravenously, after injection of 0.6 g of sodium tetrathionate in 10 cc of distilled water, and after placing

* All opinions held are those of the author and are not necessarily those of the Veterans Administration.

[1] Theis, F. V., and Freeland, M. R., *Arch. Surg.*, 1939, **28**, 191.

[2] Theis, F. V., and Freeland, M. R., *Arch. Surg.*, 1940, **40**, 190.

[3] Theis, F. V., and Freeland, M. R., *Ann. Surg.*, 1941, **113**, 411.

[4] Theis, F. V., and Freeland, M. R., *Surg.*, 1942, **11**, 101.

[5] Robinowitz, H. M., *Am. J. Surg.*, 1933, **21**, 260.

[6] Burch, G. E., *Am. Ht. J.*, 1947, **33**, 48.

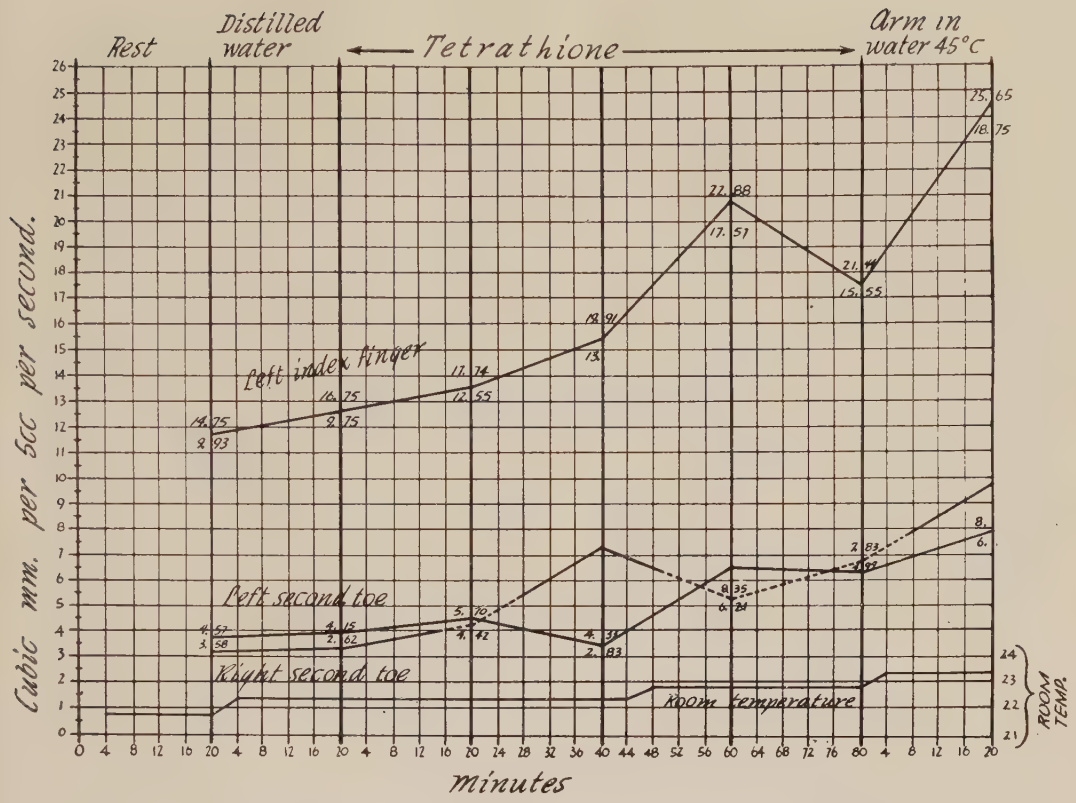

FIG. 1.

Effect of 0.6 g of sodium tetrathionate given intravenously and indirect heat on the rate of blood flow of 10 normal subjects.

one arm in water at 45°C, for a period of 20 minutes. The flow rate was measured at an average room temperature of approximately 22.5°. The room temperature varied less than plus or minus 1.1°C.

Skin temperatures were recorded from the left index finger and from the left and right great toes, using a Micromax automatic recorder. The skin temperature measurements were accurate to within plus or minus 0.5°C. The thermocouple junction was applied with Scotch Tape to prevent evaporation.

Pulse rates were recorded from the plethysmogram.

Skin temperatures and flow rates were determined simultaneously on the above mentioned subjects. The experiments were carried out on 10 normal subjects and 10 patients with arterial arteriosclerotic obliterative disease of the lower extremities who have had a left lumbar sympathectomy.

Results. The mean flow rate of 10 normal subjects at rest in the left index finger was 11.6 cu mm, per 5 cc of tissue per second, while in the left second toe and the right second toe it was 3.7 and 3.2, respectively (Fig. 1). Twenty minutes after the injection of 5 cc of distilled water the mean flow rate of the finger had increased to 12.6. The flow rates in the toes were essentially unchanged. The maximum rate of flow in the finger was reached 60 minutes after injection of sodium tetrathionate, at which time the mean flow was 20.8. The maximum flow in the lower extremities was reached in the right second toe at 40 minutes, the maximum being 7.2. Eighty minutes after the injection of sodium tetrathionate the flow rate in the finger was 17.6, while that in the right second toe and left second toe was 6.8 and 6.4, respectively. After placing the arm in water 45°C, for 20 minutes, the

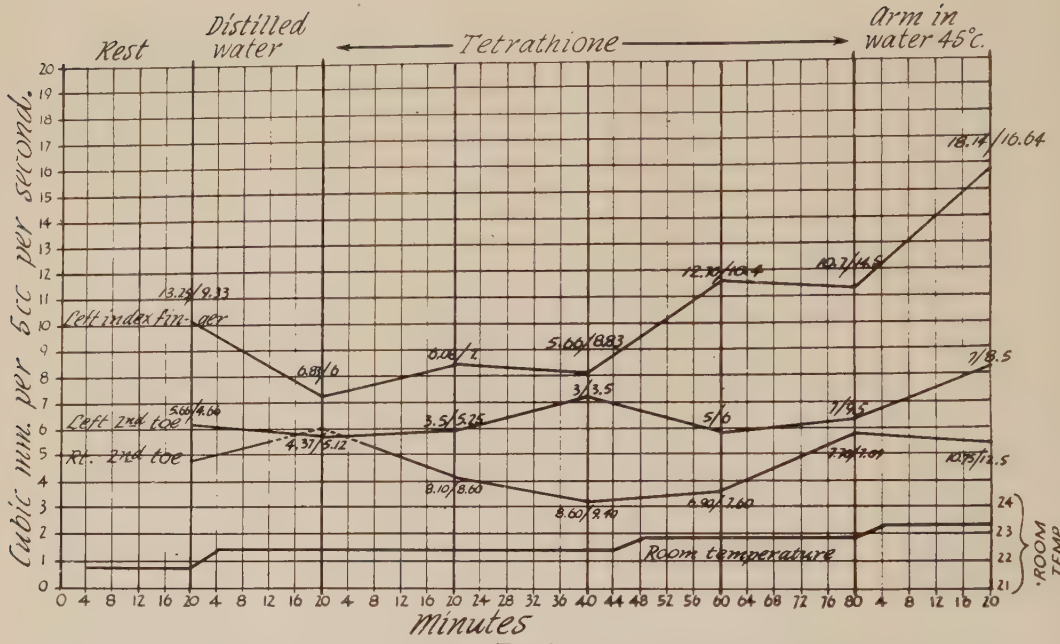

FIG. 2.

Effect of 0.6 g of sodium tetrathionate given intravenously and indirect heat on the rate of blood flow of patients with arteriosclerotic obliterative disease who have had a left lumbar sympathectomy.

mean flow rate of the finger increased to 24.5, in the right second toe to 9.8, and in the left second toe to 7.9.

Fig. 2 shows the rate of blood flow in 10 patients with arteriosclerotic obliterative disease who have had a left lumbar sympathectomy. After 45 minutes of rest the mean rate of flow in the left index finger was 10.2, in the left second toe 6.1, and in the right second toe 4.8 cu mm, per 5 cc per second. The flow rates twenty minutes after the intravenous injection of 5 cc of distilled water were 7.2, 5.8 and 5.9, respectively. The intravenous injection of 0.6 g of sodium tetrathionate produced a gradual increase in flow rate in the finger, with maximum at 60 minutes, the average being 11.6 cu mm, per 5 cc per second. The maximum flow rate in the left second toe was reached at 40 minutes, the average being 7.2 cu mm, per 5 cc per second. At this time the poorest rate of flow was detected through the right second toe, the average being 3.2 cu mm, per 5 cc per second. Eighty minutes after the injection of sodium tetrathionate the flow rates in the left index finger, the left second toe and right second toe were 11.2, 6.4, and 5.8, respectively. Twenty minutes after placing the arm in hot water at 45°C, the average flow in the finger was 15.8, in the left second toe 8.2 and in the right, 5.4.

Skin temperature measurements from the left index finger, the left great toe and right great toe were made simultaneously with the recording of flow rates (Fig. 3). After 45 minutes of rest the mean skin temperature for 10 normal subjects was 30°, 27.2° and 26.6°C, respectively. Twenty minutes after the injection of distilled water these values were 30.6, 27.2 and 26.6°C, respectively. Maximum sodium tetrathionate effect in fingers and toes was noted at 80 minutes after injection, at which time the values were 31.7, 28.8 and 27.2, respectively. Twenty minutes after placing the right arm in water up to the elbow 45°C, these values were 33.4, 31.4 and 30.5, respectively. During the course of these experiments, the room temperature increased approximately 2°C.

Skin temperatures were carried out on 10 patients with arteriosclerotic obliterative disease who have had a left lumbar sympathec-

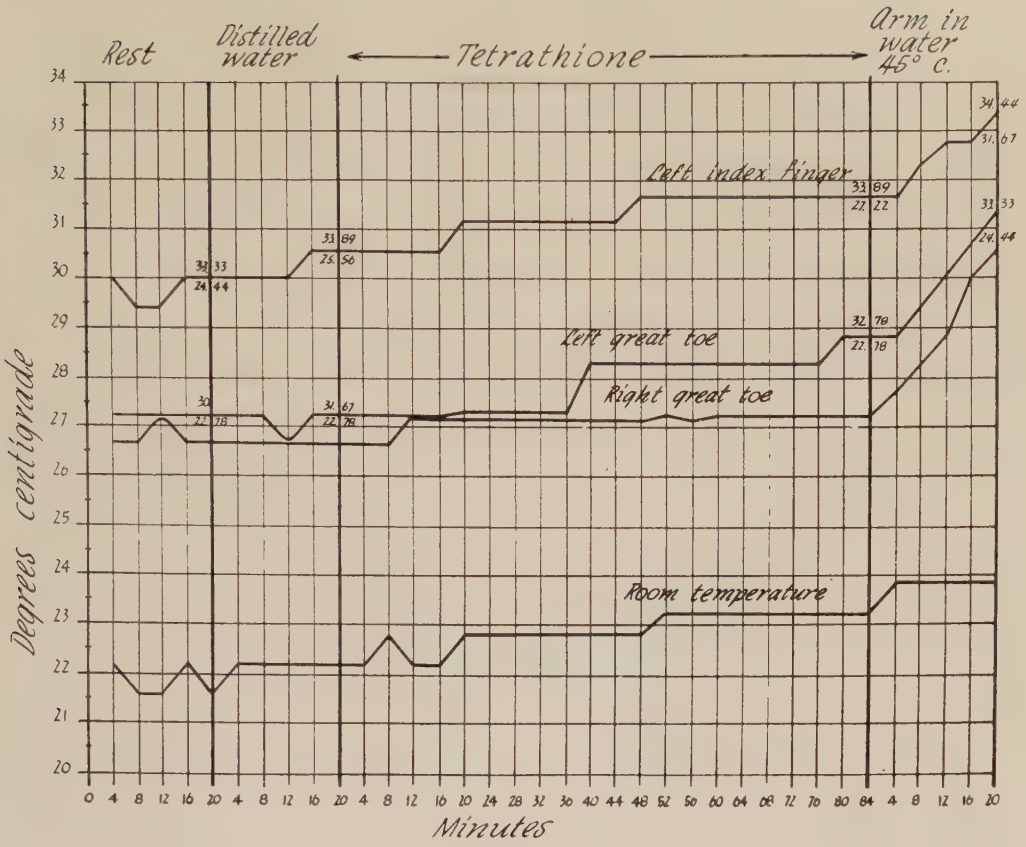

FIG. 3.

Effect of 0.6 g of sodium tetrathionate given intravenously and indirect heat on skin temperatures of 10 normal subjects.

tomy (Fig. 4). Temperature readings were made from the left index finger, left great toe and right great toe. At the end of a 45-minute rest period the temperatures were 28.8, 27.7 and 25.6°C, respectively. Twenty minutes after the injection of distilled water these values were 28.8, 27.7 and 24.4. Sodium tetrathionate produced no significant rise in skin temperature throughout an 80-minute period on the unsympathectomized lower extremity. On the sympathectomized lower extremity the rise was less than 0.5°C. Maximum skin temperature on the left index finger was reached at 68 minutes after the injection of sodium tetrathionate, at which time the temperature was 29.4°C. Eighty-four minutes after the injection of sodium tetrathionate these values were 28.3, 28.2 and 23.8, respectively. Twenty minutes after placing the right arm in water up to the elbow 45°C, these values were 32.4, 29.4 and 26.6, respectively. The room temperature increased less than 2°C, during this experiment.

The effect of the intravenous injection of 0.6 g of sodium tetrathionate in 10 cc of distilled water on the pulse rate of 10 normal subjects, is shown in Fig. 5. Ten cc of distilled water produced a fall in the pulse rate of 2 beats per minute. The lowest average pulse rate reached, after injection of sodium tetrathionate, occurred at the end of a 40-minute period, at which time the pulse rate had fallen 2 beats per minute. At the end of 60 minutes, after injection, the pulse rate returned to the pre-injection level. After placing the arm in hot water for 20 minutes there was an increase in pulse rate of one beat per minute.

Discussion. Sodium tetrathionate given intravenously to 10 normal subjects produced

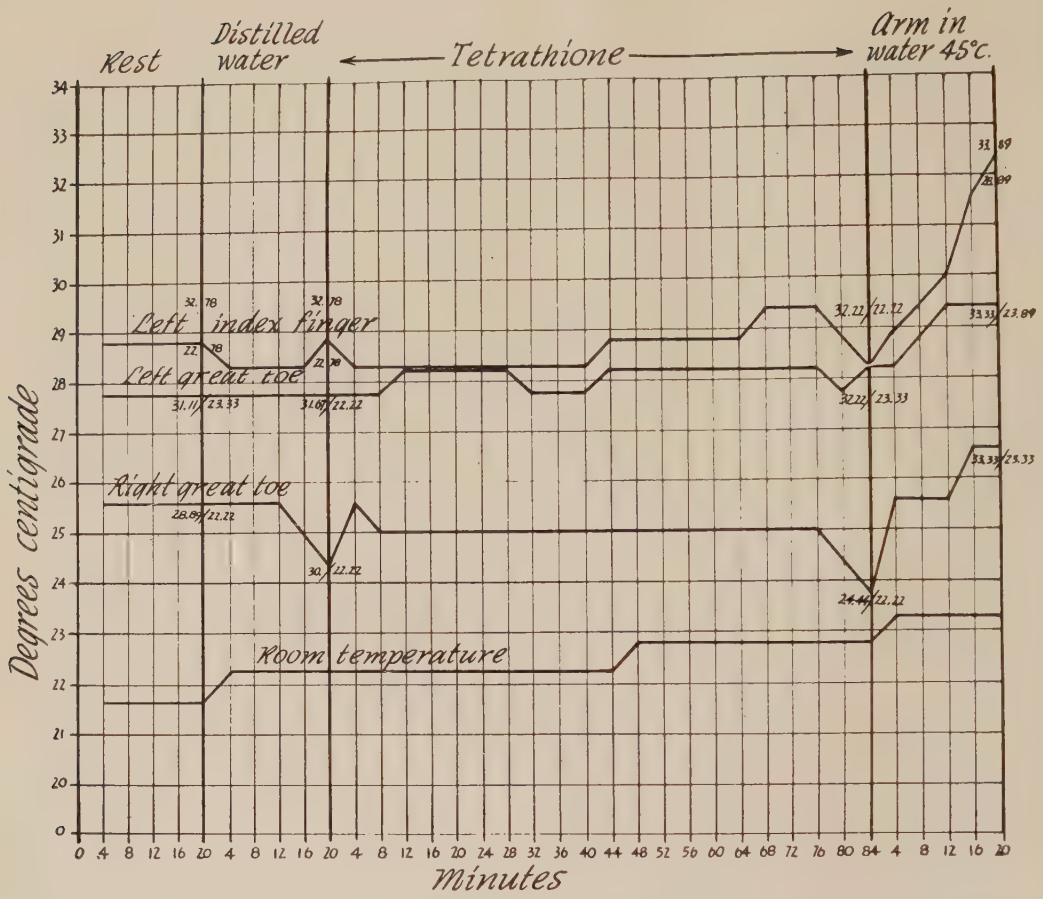

FIG. 4.

Effect of 0.6 g of sodium tetrathionate given intravenously and indirect heat on skin temperatures of 10 patients with arteriosclerotic obliterative disease who have had a left lumbar sympathectomy.

a definite but slight rise in the rate of blood flow in the fingers. In the lower extremities the flow rates were increased to a lesser extent. After placing the arm in water at 45°C, there was an additional increase in the rate of blood flow. It should be noted in these experiments that the room temperature increased slightly during the procedure. Thus, the evidence seems to indicate that sodium tetrathionate may produce a very slight increase in flow rates in normal individuals, particularly to the upper extremities. This increase in rate is not marked, however, as compared to the increased flow rates which resulted from indirect heating by placing one arm in water 45°C. In all instances, the arm was placed in water to a level somewhat below the elbow. Subsequent experiments have shown that incomplete dilatation is ordinarily produced by this method. The rate of flow can be augmented in individuals by placing the arm in water 45°C, with the level of the water well above the elbow. A still greater rate of flow is recorded when both arms are placed in water above the elbow. This is particularly true when the patient is heavily covered with blankets. Thus, sodium tetrathionate must be considered relatively ineffective in increasing the flow rates in these individuals. The skin temperatures of these 10 normal subjects increased only slightly, approximately 2° after the use of sodium tetrathionate. As the room temperature also increased slightly during these experiments, it is felt that sodium tetrathionate had no definite effect upon the skin temperatures.

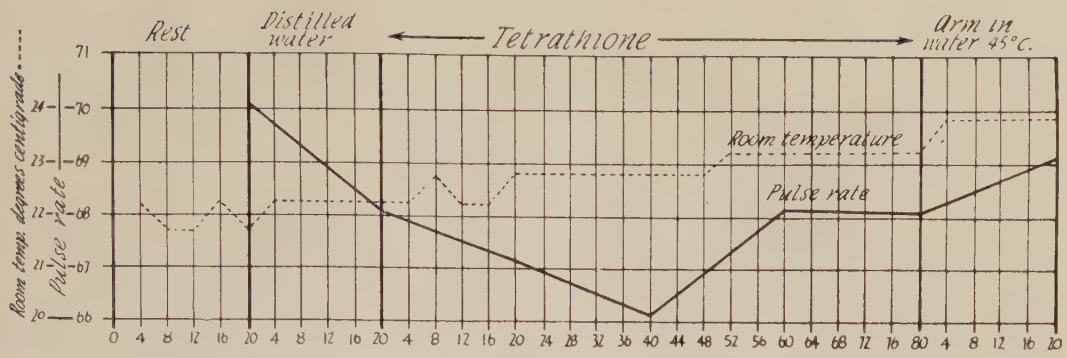

FIG. 5.
Effect of 0.6 g of sodium tetrathionate given intravenously and indirect heat on the pulse rate of 10 normal subjects.

Again placing the arm in water 45° C, below the elbow produced a greater increase in the skin temperatures than that produced by sodium tetrathionate. Here again, placing both arms in water at this temperature above the elbow often produces a greater degree of temperature rise than that following the insertion of a single arm in water below the elbow. The 10 patients with arteriosclerotic obliterative disease showed essentially no significant increase in flow rates to the lower extremities following the injection of sodium tetrathionate. The rate of flow in the finger increased somewhat after the use of intravenous sodium tetrathionate. The increase was less than that seen in the normal group. Here again, indirect heating increased the flow rates more markedly than did sodium tetrathionate. Skin temperature measurements showed a slight increase in skin temperature of the left index finger after sodium tetrathionate. There was no significant increase in skin temperature of the toes. After the indirect heating the skin temperatures of the fingers and toes increased. This would indicate that sodium tetrathionate is not useful in increasing the rate of blood flow nor in raising the skin temperature in the lower extremities in individuals with arteriosclerotic obliterative disease in either an unsympathectomized lower extremity or in a sympathectomized lower extremity.

It should be pointed out that in determining the rate of blood flow, the occluding cuff was placed at the wrist or ankle. If the cuff had been placed at the base of the finger or at the base of the toe, the flow rates would have been approximately 3 times greater.[7]

Lastly, it appears that sodium tetrathionate produces a very slight slowing of the pulse rate which occurs approximately 40 minutes after injection. During these injections no toxic effects were encountered. In all instances the drug was injected very slowly. Thus, it seems that if sodium tetrathionate is of value in the treatment of peripheral vascular disease, it must act by mechanisms other than by increasing the rate of blood flow.

Summary. The rate of blood flow, skin temperature, and pulse rates were determined after the intravenous injection of 0.6 g of sodium tetrathionate. In 10 normal subjects the rate of blood flow and skin temperature increased slightly in the left index finger. In the lower extremities the rates of flow increased to a lesser extent. Indirect heating produced a greater rate of flow in the upper and lower extremities and increased the temperature in the upper and lower extremities to a greater degree. In 10 subjects with arteriosclerotic obliterative disease who have had a left lumbar sympathectomy, the rate of flow and skin temperatures through the upper extremities were increased slightly. However, there was no significant change in the lower extremities. Indirect heating was more efficacious in increasing the flow rate and skin temperature than was the sodium tetrathionate. The pulse rate was slowed slightly, the maximum slowing occurring 40 minutes after injection.

[7] Goetz, Robert H., *Am. Ht. J.*, 1946, **31**, 146.

16384 P

Evidence for an Adrenergic Component in the Nervous Mechanism of Sweating in Man.*

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In the course of an investigation on the vasomotor effects of Dibenamine† (N,N-dibenzyl-β-chloroethylamine hydrochloride), a new adrenergic-blocking agent, it was found that spontaneous palmar sweating in man was inhibited. The interpretation of this observation appeared to be at variance with the known concept of the strictly cholinergic innervation of these glands.

The apparent discrepancy between our observation and the classical concept of the nature of the nervous supply to the sweat glands prompted this investigation.

Methods and Material. A colorimetric method, developed by Silverman and Powell,[1] was used for determining the presence and the amount of sweating. Prints of the sweat glands were obtained on paper treated with tannic acid. The tannic acid reacts with the iron of ferric chloride painted on the skin, to form a stain on the paper ranging from gray-blue to blue-black. The size and intensity of the resulting pattern are directly proportional to the amount of sweat secreted.

Sweat prints of the palms were taken in all instances. Occasionally those of the forehead, cheeks, forearm and plantar region of the feet were also taken. In conjunction with the study on sweat, measurements of skin temperature of the head and the upper and lower extremities were recorded in all cases. These determinations were made under basal conditions. The environmental temperature ranged from 72° to 76°F, with a fairly constant humidity. Sweat prints and thermometric changes were recorded before and after the administration of Dibenamine. The latter was administered by the intravenous route, at the dosage of 5 mg per kg of body weight. The exact details of the method of administration were reported in a previous publication.[2]

The anhidrotic effects of this adrenergic-blocking drug were studied 52 times in 24 subjects, using the colorimetric method. In addition, at the early stage of this investigation, simple clinical observations of the suppression of sweating were made 8 times in 2 other subjects. Eleven of these subjects had essential hypertension, 8 had various vascular conditions, and 7 had various neurological syndromes.

Results. (a) In subjects exhibiting intense or strong spontaneous palmar sweating, Dibenamine administration was usually followed either by total or partial suppression of sweating. In subjects with moderate or faint sweat response patterns, Dibenamine induced in most instances a complete suppression of sweating. Concomitant measurements of skin temperature showed in each case a maximum vasodilation of the cutaneous vessels of the hands and fingers (90° to 94°F).

(b) Intravenous injection of neosynephrine (0.5-1.0 mg) induced moderate sweating. After Dibenamine, neosynephrine remained without effect on the sweat function. Mecholyl (25 mg) given subcutaneously after Dibenamine, induced profuse sweating, mostly on the face and on the dorsum of the hands.

When vomiting, a reflex vagal phenomenon, followed the administration of Dibenamine it was usually accompanied by marked or

* This investigation was supported by the Peripheral Vascular Diseases Research Fund.

† Trademark, Givaudan-Delawanna, Inc. Dibenamine was supplied by the Givaudan-Delawanna, Inc., Delawanna, N.J., through the courtesy of Dr. W. Gump.

1 Silverman, J. J., and Powell, V. E., *Am. J. Med. Sc.*, 1944, **208**, 297.

2 Haimovici, H., and Medinets, H. E., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 163.

profuse spontaneous sweating. Within 15 to 30 minutes after cessation of vomiting, however, the anhidrotic effect of Dibenamine became again apparent.

Discussion. The known fact that the sweat glands, although under the control of the sympathetic nervous system, respond to parasympathetic drugs, has long been a puzzle. Dale and Feldberg[3] have shown in the cat that the fibers governing these glands were cholinergic.

In man, parasympathetic drugs alone were known to promote or suppress sweat secretion. Certain types of sweating in man, however, could receive no satisfactory explanation on the basis of the concept of the exclusive cholinergic innervation. Indeed "cold sweat" due to psychic stimulation or that accompanying the so-called sympatho-adrenal attacks in pheochromocytoma, are such examples. In the latter case, there is ample evidence to show that the clinical manifestations are mediated through circulating adrenaline.[4,5] The mechanism whereby sweating is induced under these circumstances does not seem to be in harmony with the cholinergic concept.

The inhibition of sweating induced by Dibenamine appears to be an adrenergic-blocking phenomenon. Indeed, it has been shown, both in animals and man, that Dibenamine inhibits the pressor effect of epinephrine, neosynephrine or that following stimulation of adrenergic fibers.[2,6,7] In addition, myosis in man caused by the administration of Dibenamine is not reversible by epinephrine or paredrine while it is by atropine.[8] These facts indicate that Dibenamine is a specific adrenolytic and sympatholytic drug. It can therefore be assumed that the suppression of sweating by Dibenamine is truly an adrenergic-blocking effect.

The production of sweat by sympathomimetic drugs is a controversial matter. While in certain animals (horse, sheep) epinephrine induces marked sweating,[9,10] in man, it is known to fail to do so. Most investigators, with the exception of Freund[11] are in agreement on this point. However, sweating in man is known to occur after ephedrine[12] and benzedrine.[13]

In this study neosynephrine, a sympathomimetic drug having many similarities to epinephrine, induced moderate sweating. The effect of sympathomimetic agents on sweat function is under further investigation.

The production of sweating by mecholyl, after Dibenamine, indicates that the cholinergic fibers remained unaffected by the latter. This is another evidence of the specific adrenergic-blocking action of Dibenamine.

From the data presented above it appears that, in addition to the known cholinergic fibers supplying the sweat glands, there is also an adrenergic component in the nervous mechanism of sweating in man.

I wish to express my thanks to Drs. Louis Leiter (Medical Division) and H. Houston Merritt (Neuro-Psychiatric Division) for permission to use patients from their respective divisions for this investigation.

The assistance of Dr. H. E. Medinets is acknowledged.

3 Dale, H. H., and Feldberg, W., *J. Physiol.*, 1934, **82**, 121.

4 Beer, E., King, F. H., and Prinzmetal, M., *Ann. Surg.*, 1937, **106**, 85.

5 Goldenberg, M., Snyder, C. H., and Aranow, H., Jr., *J. A. M. A.*, 1947, **135**, 971.

6 Nickerson, M., and Goodman, L. S., *J. Pharm. and Therap.*, 1947, **89**, 167.

7 Haimovici, H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 486.

8 Haimovici, H., unpublished data.

9 Muto, K., *Mitt. med. Fak. Univers. Tokyo*, 1916, **15**, 365 (in Kuno, Y., 9a).

9a Kuno, Y., *The Physiology of Human Perspiration*, London, J. & A. Churchill, Ltd., 1934, 278 pp.

10 Langley, J. N., and Bennett, S., *J. Physiol.*, 1923, **57**, 121.

11 Freund, E., *Wien. klin. Wchnschr.*, 1920, **33**, 1009.

12 Chen, K. K., and Schmidt, C. F., *Medicine*, 1930, **9**, 1.

13 Wilbur, D. L., MacLean, A. R., and Allen, E. V., *J. A. M. A.*, 1937, **109**, 549.

16385

Action of Prostigmine, Carbaminoyl-choline (Doryl) and Acetyl-B-methyl-choline (Mecholyl) on Intestine of a Cladoceran.

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From the Department of Biology, Bard College.

In a previous work it was demonstrated by Obreshkove[1,2] that it is possible to study the physiological action of acetylcholine and physostigmine by observing their effects on the intestine and the heart of *Daphnia magna*. The results obtained were of such a nature as to suggest strongly some known facts pertaining to the role which these drugs have been said to play in the transmission of nervous impulses. Although there has not been demonstrated in Cladocera anything which corresponds morphologically to the autonomic nervous systems in vertebrates, if the intestine of *Daphnia magna* is touched with a fine glass needle at the bend of the digestive tube where the intestine enters the stomach, the heart immediately stops beating and the posterior portion of the digestive tube commences to exhibit powerful intestinal contractions. The heart in this respect behaves like the inhibition produced after electrical excitation of the vagus nerve in vertebrates. These and other observations lead us to extend this type of work to other drugs which have been said to play a similar role in the body as acetylcholine and physostigmine. In this work 3 more recently introduced drugs were utilized for a study, namely, carbaminoyl-choline (Doryl), acetyl-B-methyl-choline (Mecholyl) and prostigmine. The nature of their action was compared with that of acetylcholine and physostigmine. Our interest is primarily centered on the comparative physiological evaluation of these chemicals with regard to their stability, potency of action, and their pharmacological effects as observed on the intestine of the *Simocephalus vetulus*, a cladoceran. In spite of the comparatively low structural organization of this organism, these drugs induce such unmistakably dramatic effects on the musculature of the intestine that their action is remarkably similar to the therapeutic value ascribed to them in the treatment of atony and intestinal distention.[3]

Methods. In this work *Simocephalus vetulus* young in their second instar were employed. The method of rearing this animal and other cladocera, and the method employed in the selection of animals which are in the same stage of development have been described elsewhere.[4] The animals were subjected to experimentation separately; in each case a single individual was transferred to a micro culture slide for treatment with the specific drug employed. Animals which were found to be in an excited state (probably due to unfavorable culture media) were not accepted for the experimental work. Fresh solutions of the drugs were prepared daily, usually just before their utilization. The dilutions recorded in this paper are expressed in per cent concentration.

Results. When a *Simocephalus vetulus* young is treated with prostigmine, the muscular peristaltic and antiperistaltic contractions of the intestine become extremely violent. When the drug becomes effective, it exhibits an abrupt, powerful contractile wave of considerable amplitude and the effects persist for some hours thereafter. Prostigmine showed graded action for a wide range of concentrations, namely, a definite relation was established between the time which elapses from the application of the drug to the onset of the initial vigorous peristaltic wave and the concentrations employed.

The time required to induce the initial

1 Obreshkove, V., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 427.

2 Obreshkove, V., *Biol. Bull.*, 1941, **81**, 105.

3 Fraser, F. R., *Brit. Med. J.*, 1938, pp. 1293-1299.

4 Obreshkove, V., *Physiol. Zool.*, 1930, **3**, 271.

TABLE I.
Onset of Vigorous Intestinal Contractions in *Simocephalus vetulus* After Treatment with Prostigmine of Various Concentrations.

Prostigmine 1×10^{-1} Sec.	Prostigmine 1×10^{-2} Min.	Prostigmine 1×10^{-3} Min.	Prostigmine 1×10^{-4} Min.	Prostigmine 1×10^{-7} Min.
35	2.5	6.1	21	86
55	1.9	5.6	25	78
60	2.7	9.1	11	63
40	1.2	6.4	24	88
63	1.6	5.8	35	72

effect of the drug varied from less than one minute for an abnormally high prostigmine concentration (1×10^{-1}) to more than one hour for more dilute solutions (1×10^{-7}). This graded action is well illustrated in Table I. The analogue of prostigmine, namely, physostigmine, was observed to produce a similar action on the intestine of *Daphnia magna*.[5] It stimulated the intestinal peristalsis in *Daphnia magna* as well as in *Simocephalus vetulus* as powerfully as prostigmine. However, the period which elapsed from the application of the drug to the onset of the vigorous peristaltic waves was found to be much longer for prostigmine than for physostigmine (Table V) for the same concentration of the drugs. With every concentration of these two drugs, it was observed that the amplitude of the peristaltic waves increased with the lapse of time.

TABLE II.
Onset of vigorous intestinal contractions in *Simocephalus vetulus* after treatment with acetylcholine (1×10^{-5}) and also the time of action of the same concentration of the drug following the administration of prostigmine (1×10^{-5}) for 2 minutes.

Acetylcholine 1×10^{-5} min.	Acetylcholine 1×10^{-5} after treatment with prostigmine (1×10^{-5}) sec.
12.3	35
15.0	40
13.0	38
19.0	35
12.8	45
14.0	60
17.0	44

Prostigmine and Acetylcholine. Prostigmine causes in *Simocephalus vetulus* intensification and prolongation of the effects of acetylcholine. Individuals which were treated with prostigmine prior to the application of acetylcholine gave a reaction time for the onset of the characteristic effects which was considerably less than the time required for the action of acetylcholine and prostigmine alone. This was found to be true for a wide range of concentrations of these two drugs. The reduction in the time of action of acetylcholine due to prior application of prostigmine was found to be considerable. When acetylcholine (1×10^{-5}) was administered to the animal alone the time of action was found to be on the average about 15 minutes. Prostigmine reduced this period to less than one minute (Table II). This action of prostigmine as well as physostigmine has been explained on the basis that, in mechanisms in which the transmission of nervous impulses have been associated with acetylcholine, they delay the destruction of acetylcholine by cholinesterase. In view of the demonstration by Artemov and Mitropolitanskaja[6] of the presence in whole daphnia of an acetylcholine-like substance and cholinesterase in the hemolymph of crustacea, as well as the demonstration of the occurrence of acetylcholine in the nervous tissue in crustacea, the observations recorded in Table II acquire added significance. In man prostigmine stimulates intestinal peristalsis as powerfully as physostigmine.[7] Prostigmine has been recommended by the manufacturers for therapeutic use as an intestinal stimulant in the case of post-operative intestinal atony,

[5] Obreshkove, V., *Biol. Bull.*, 1941, **81**, 105.

[6] Artemov, N. M., and Mitropolitanskaja, R. L., *Bull. de Biol. ed. de Méd. Expér. U. S. S. R.*, **5**, 378.

[7] Ammon, A., *Arch. Ges. Physiol.*, 1933, **233**, 486.

TABLE III.
Onset of Vigorous Intestinal Contractions in *Simocephalus vetulus* After Treatment with Doryl* of Various Concentrations.

Doryl 1×10^{-3} Sec.	Doryl 1×10^{-4} Sec.	Doryl 1×10^{-6} Sec.	Doryl 1×10^{-9} Sec.	Doryl 1×10^{-12} Sec.
26	32	20	20	21
24	27	27	25	30
55	36	31	28	25
30	39	28	32	40
28	45	24	35	30
35	28	35	24	33

* Doryl—trade name for carbaminoyl-choline.

and it appears that its effect may be due to the potentiation of the acetylcholine effects normally occurring in the body.[8]

Carbaminoyl-choline (*Doryl*). Since its introduction by Kreitmair,[9] carbaminoyl-choline has been observed to produce the same qualitative effects as acetylcholine.[9] In the mammal it has been shown to act typically as a mimic of parasympathetic stimulation, and its effects, as in the case of acetylcholine, are inhibited by atropine. Carbaminoyl-choline produces the same effects on the intestine of the *Simocephalus vetulus* as was demonstrated for acetylcholine, prostigmine and physostigmine. It differs from acetylcholine primarily in its greater stability, in its more powerful action and in its greater effectiveness as judged by the time which elapses between the application of the drug and the onset of the characteristic effect. Various concentrations of this drug were employed in the experimental work, ranging from concentrations of 1×10^{-3} through 1×10^{-12} (Table III). The drug was so effective and the characteristic effect was produced with such rapidity that the graded action so characteristic of acetylcholine and prostigmine could not be demonstrated within the range of concentrations employed in this work. Very dilute solutions (1×10^{-12}) produced the characteristic effect in approximately the same length of time as that of considerably stronger solutions (Table III). The action of a comparatively weak solution of carbaminoyl-choline required a high concentration of atropine (1×10^{-1}) to abolish its effects. Even after the application of very high concentrations of atropine the effects of carbaminoyl-choline persisted in some cases for over 2 hours or more before they were completely abolished. The effects of stronger solutions of acetylcholine (1×10^{-3}), on the other hand, were abolished in the majority of cases in less than 2 minutes by a much weaker solution of atropine (1×10^{-5}). The antagonistic effect of atropine on acetylcholine and carbaminoyl-choline respectively is shown in Table IV. It is evident from an inspection of this table that atropine acts more slowly and less effectively in antagonizing the action of carbaminoyl-choline. It has been said concerning this drug that it is not susceptible to destruction by cholinesterase and that its activity is not affected by antiesterase drugs such as physostigmine and prostigmine.

Acetyl-B-methyl-choline (*Mecholyl*). The intestine of the *Simocephalus vetulus* after treatment with acetyl - B - methyl - choline straightens out as a result probably of the much increased muscle tonus of the organ. The lumen of the entire digestive tube decreases and there appears a sphincter-like constriction just posterior to the rectal region. These effects were observed with all the dilutions employed, and the time of their appearance was found to vary, as in the case of the graded action demonstrated for acetylcholine and prostigmine, with the strength of the drug employed. Prior to the formation of the rectal constriction, much of the contents of the digestive tube is emptied in a few seconds after the introduction of the drug due to the

[8] Fraser, F. R., *Brit. Med. J.*, 1938, pp. 1349-1354.

[9] Kreitmair, H., *Arch. ex. Path. Pharmak.*, 1932, **164**, 346.

TABLE IV.

Onset of Vigorous Intestinal Contractions in *Simocephalus vetulus* After Treatment with Acetylcholine, Doryl,* and Mecholyl† and the Time of Abolishing the Characteristic Effects by Atropine.

Acetylcholine 1×10^{-3} Sec.	Atropine 1×10^{-5} Sec.	Doryl 1×10^{-4} Sec.	Atropine 1×10^{-1} Min.	Mecholyl 1×10^{-1} Sec.	Atropine 1×10^{-8} Sec.
21	95	50	78	25	134
25	60	45	48	40	75
23	69	60	110	33	82
26	133	46	117	35	240
27	90	42	134	28	317
29	144	35	94	34	340

* Doryl—trade name for carbaminoyl-choline.
† Mecholyl—trade name for acetyl-B-methyl-choline.

TABLE V.

A Comparative Study of the Time of Action in Inducing Vigorous Intestinal Contractions in *Simocephalus vetulus* After Treatment with the Same Concentration (1×10^{-4}) of Doryl, Acetylcholine, Physostigmine, Prostigmine, and Mecholyl.

Doryl 1×10^{-4} Sec.	Acetylcholine 1×10^{-4} Min.	Physostigmine 1×10^{-4} Min.	Prostigmine 1×10^{-4} Min.	Mecholyl 1×10^{-4} Min.
27	8.2	9.5	21	26
42	7.8	8.8	26	14
52	8.6	9.0	11	24
46	7.8	9.2	24	27
32	10.0	9.0	35	40
35	7.6	8.5	25	36

very powerful initial peristaltic waves. There was also observed a very rapid palpitation of the anal portion of the digestive tube posterior to the constriction, but no relaxation of the sphincter effect appeared as long as the intestine was under the influence of the drug. Starr[10] reports similar effects induced by Doryl and Ritvo[11] by Mecholyl on the human esophagus and stomach as those described above for Mecholyl on the intestine of the *Simocephalus vetulus*.

In contrast with carbaminoyl-chloride a very clear antagonism was found to exist between acetyl-B-methyl-choline and atropine. A very weak atropine solution (1×10^{-8}) very quickly overcame the effects of abnormally high concentration of acetyl-B-methyl-choline (1×10^{-1}). This is in marked contrast with the period required to abolish the effects produced by relatively weak carbaminoyl-choline by strong concentrations of atropine (Table IV). The time of action of a wider range of concentrations of atropine, as well as the time of the re-establishment of strong intestinal contractions by acetylcholine following atropine, has been recorded for *Daphnia magna*.[12]

The existence of this marked antagonism between acetyl-B-methyl-choline and atropine was further demonstrated. In a series of 5 experiments a preliminary application of atropine 1×10^{-5} for 5 minutes prevented the appearance of the characteristic effect ascribed to this drug. Myerson, Loman and Dameshek[13] have demonstrated that acetyl-B-methyl-choline in the human induces secretory and vaso-motor effects. They, too, have observed that small doses of atropine when given prior to the administration of acetyl-B-methyl-choline prevented the appearance of the characteristic Mecholyl effects.

The comparative physiological and pharmacological effects of acetylcholine, prostigmine, carbinoyl-choline, and acetyl-B-methyl-choline are shown in Table V. From an inspection of

[10] Starr, J., *Am. J. Med. Sc.*, 1937, **193**, 393.
[11] Ritvo, M., *Am. J. Roentgen.*, 1936, **36**, 868.
[12] Obreshkove, V., *Biol. Bull.*, 1941, **81**, 105.
[13] Myerson, A., Loman, J., and Dameschek, W., *Am. J. Med. Sci.*, 1937, **193**, 198.

the table, it is seen that, when individuals are subjected to drugs of the same concentration (1×10^{-4}), Doryl produces vigorous intestinal contractions in the shortest period of time. Acetylcholine and physostigmine come next in order of effectiveness and appear to be equally potent. Prostigmine and acetyl-B-methyl-choline are the least effective of all the drugs utilized in this experimental work.

Summary. 1. Prostigmine, acetylcholine, carbaminoyl-choline and acetyl-B-methyl-choline produce in *Simocephalus vetulus* vigorous intestinal contractions.

2. The period which elapses between the addition of the drugs and the onset of the characteristic effect is definitely dependent, with the exception of carbaminoyl-choline, on the concentration of the drug.

3. Carbaminoyl-choline did not exhibit a graded action within the range of concentrations employed in this work.

4. Atropine blocks the action of the drugs employed in this work. However, it acts more slowly and less effectively in antagonizing the action of carbaminoyl-choline.

5. Prostigmine causes intensification and prolongation of the effects of acetylcholine and it reduces considerably the time which elapses between the administration of acetyl-choline and the appearance of the vigorous intestinal contractions.

6. In contrast with carbaminoyl-choline, a marked antagonism was found to exist between acetyl-B-methyl choline and atropine.

7. Carbaminoyl-choline differs from acetyl-choline primarily in its greater stability and more powerful action.

8. The action of prostigmine, acetyl-choline, carbaminoyl-choline and acetyl-B-methyl-choline is remarkably similar in certain respects to the action ascribed to them on the digestive tube for a number of mammals.

16386 P

A Latent Pneumotropic Pasteurella of Laboratory Animals.

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Laboratory mice are known to harbor a variety of latent pneumotropic agents including bacteria,[1] pleuropneumonia-like organisms[2,3,4] and viruses.[5–12] Increasing interest in human respiratory infections has led to many attempts to adapt agents causing such diseases to small laboratory animals. Blind passage of lung material by the respiratory route frequently leads to the production of lung lesions in the experimental animals due to the activation of latent agents. Eliminating such a possibility is an important step in ascertaining the relationship of the agent studied to the lesions produced.

In the course of attempts to adapt to Swiss

[1] Dingle, J. H. Biology of the Laboratory Mouse, The Blakiston Co., Phila. 1941. Chap. 12, pp. 380-474.

[2] Sullivan, E. R. and Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 620.

[3] Edward, D. G. F., *J. Path. and Bact.*, 1940, **50**, 409.

[4] Edward, D. G. F., *J. Path. and Bact.*, 1947, **59**, 209.

[5] Andrewes, C. H. and Glover, R. E., *Brit. J. Exp. Path.*, 1945, **26**, 379.

[6] Dochez, A. R., Mills, K. C., and Mulliken, B., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 683.

[7] Gonnert, R., *Klin. Wochenschr.* 1941, **20**, 76.

[8] Gordon, F. B., Freeman, G., and Clampit, J. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 450.

[9] Heinzmann, K., *Klin. Wochenschr.*, 1941, **20**, 910.

[10] Horsfall, F. L. and Hahn, R. G., *J. Exp. Med.*, 1940, **71**, 391.

[11] Nelson, J. B., *J. Exp. Med.* 1937, **65**, 833.

[12] Nigg, C., *Science*, 1942, **95**, 49.

mice an agent causing a minor respiratory illness in human volunteers[13] pneumonic lesions were encountered in animals inoculated intranasally with mouse lung material in serial passage. Some animals showed only a transient illness between the 4th and 15th days after inoculation; others died. In about 60% of the mice there was a grey consolidation of the upper lobes, commonly on the left, often with a yellow necrotic nodular center. Histologically the lungs showed a mononuclear exudate filling alveoli and bronchi with large necroses in the center of the involved areas. All other organs appeared normal.

In smears from consolidated lung areas, there were occasional short rods stained red by Macchiavello's method, and poorly by Gram's. From the lesions a short coccoid bipolar Gram-negative rod was cultured regularly on simple media. Cultural and biochemical characteristics suggested a close relationship of this organism to the Pasteurella group. Similar Gram-negative rods have been observed by others[14,15,16] in the lungs of mice.

At first the disease could be reproduced only by intranasal administration of infected lungs, but not by bacteria-free filtrates or pure cultures of the Pasteurella. Yet all attempts failed to demonstrate other agents which might be responsible for the process either alone or in combination with the isolated bacteria.

With serial animal passage, more of the inoculated mice became ill and showed lung lesions. In the 14th passage 85% died. The Pasteurella recovered from these animals was virulent so that intranasal instillation of undiluted 18-hour broth (about 5 x 10^6 bacteria) regularly killed 21-day-old mice with widespread bronchopneumonia in 2-5 days. Adult mice were somewhat more resistant.

A survey of Swiss mice from the breeding colonies of the National Institute of Health, the Army Medical School,* and 2 commercial breeders indicated that similar organisms could be recovered from 70-95% of the lungs ground with alundum in infusion broth. The same Pasteurella was also recovered from 70% of the lungs of normal guinea pigs and white rats. The organism seems to be acquired early in life, perhaps transmitted from the mother by the respiratory route, because it was recovered 24 hours after birth in the respiratory tract of 50% of newborn mice.

Biochemical and morphological studies supported the belief that this organism is a member of the Pasteurella group. Serological study of 26 strains showed them to belong to one group, with only minor antigenic differences between strains, but quite distinct from such related organisms as *P. pesti, P. pseudotuberculosis, P. multocida, Hemophilus* species, *A. bronchisepticus*.

Virulent strains of the organism resulting from 21 animal passages remained remarkably pneumotropic. For 21-day-old mice the LD_{50} was 5 x 10^5 organisms and the ID_{50} (producing pulmonary consolidation) about 10^4 bacteria, administered intranasally under ether anesthesia. Ten times larger doses failed to produce lesions or death if administered intravenously, intraperitoneally, or subcutaneously to young mice, rabbits, guinea pigs, cotton rats, white rats, hamsters, and chickens. After intranasal inoculation of sublethal doses into young mice the Pasteurella could be recovered for 4 days from lungs, liver, spleen, kidney, and heart blood. During this period the animals were hunched, ruffed, dyspneic, cyanotic, anorexic and "chattering." Later the symptoms of illness disappeared and bacteria were found only in the lung lesions, often persisting for 2 months or longer.

With intracerebral inoculation of mice the LD_{50} was 10^4 organisms, and death occurred within 24-48 hours from a brain abscess without systemic dissemination of bacteria.

The following findings seem to establish the relationship of this Pasteurella to the described disease of mice:

13 Topping, N. H. and Atlas, L. T., *Science*, 1947, **106**, 636.

14 Andrewes, C. H., Laidlaw, P. P., and Smith, W., *Lancet*, 1934, **227**, 859.

15 Hoyle, L., *J. Path. and Bact.*, 1935, **41**, 163.

16 Kairies, A. and Schwartzer, K., *Zentr. Bakt. Abt. 1. Orig.*, 1936, **137**, 351.

* Through the courtesy of Dr. J. E. Smadel.

1. Pure cultures of the virulent organism regularly produce the disease.

2. Mice immunized with living avirulent or killed bacterial vaccines withstand massive challenge infection.

3. Infected mice can be cured by treatment with streptomycin 0.1 g/kg/day for 4 days. The Pasteurella is inhibited by 2-4 units streptomycin/cc of medium. If mixed with infectious lung suspensions streptomycin regularly prevents the lesions and disease due to this Pasteurella.†

4. Specific antisera protect against the disease after inoculation with infectious lung material or cultures.

A full account of this Pasteurella and its host-parasite relationships will be published later.

Summary. A latent pneumotropic Pasteurella was isolated from the lungs of healthy laboratory animals. In serial passage of lung material this organism was responsible for striking lung lesions, disease and death of Swiss mice. Streptomycin cured infected animals and could be used to prevent transfer of the latent Pasteurella in serial passage of lung material.

† The Gram-negative organism removed by streptomycin from passage lung material by McKee and Hale (*Science,* 1947, **105**, 41) may have been identical with the Pasteurella described here.

16387

Effect of Continued Desoxycorticosterone Administration in Hypertensive Subjects.*

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In hypertensive individuals, as compared to those with normal blood pressure, an accelerated pressor response has been observed following the subcutaneous administration of desoxycorticosterone acetate (DCA).[1] Studies are now reported concerning the effects of this steroid on the blood pressure, electrolyte and fluid balance when injected into patients with hypertensive vascular disease for longer periods.

Methods. Two men and 3 women with uncomplicated hypertensive vascular disease were observed on the wards of the Presbyterian Hospital. The criteria in selection of patients and the methods employed were identical to those previously described,[2] all subjects being maintained on a constant dietary, fluid and sodium chloride intake. Following a 3-week baseline period, DCA† was injected subcutaneously in doses of 10 mg daily for 30 days in 4 patients; a fifth received 20 mg daily for 50 days.

Results. The expected increase in weight and hemodilution which follow DCA injection were noted, together with slight reduction in urinary volume and evidence of sodium and chloride retention.[1-3] These alterations were, however, limited to the first 7-10 days of treatment, and the weight curve, hematocrit, serum protein concentration, urinary volume, 24-hour urine sodium and chloride values returned to baseline levels by the end of the second week. Serum volume, measured with the blue dye T.1824 at weekly intervals

* Aided by grants from the National Institute of Health (U.S.P.H.S.) and the Albert and Mary Lasker Foundation.

1 Perera, G. A. and Blood, D. W., *Ann. Int. Med.,* 1947, **27**, 401.

2 Perera, G. A. and Blood, D. W., *J. Clin. Invest.,* 1947, **26**, 1109.

† Desoxycorticosterone acetate (Doca) was supplied through the generosity of Dr. K. W. Thompson of Roche-Organon, Inc., Nutley, N. J.

3 Clinton, M., Jr., and Thorn, G. W., *Bull. Johns Hopkins Hosp.,* 1943, **72**, 255.

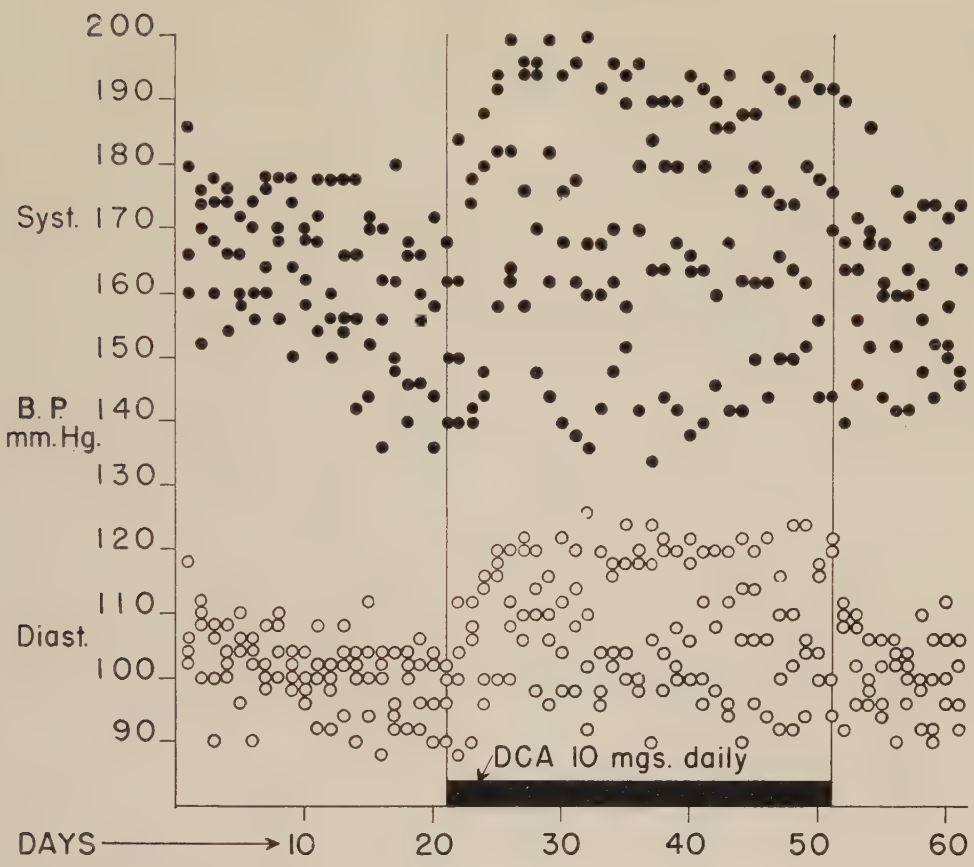

FIG. 1.

Effect of desoxycorticosterone acetate on the blood pressure of four patients with hypertensive vascular disease.

in one patient, rose from 2360 cc to 2530 cc after one week of DCA, then fell to 2320 cc by the end of the second week. Weekly determinations of serum carbon dioxide content revealed rising values with reciprocal decreases in chloride concentration. The urea nitrogen and sodium concentration of the serum showed no significant changes, but in all instances there was a progressive decline in serum potassium to values as low as 2.3 milliequivalents per liter (Table I). The increase in serum sodium concentration observed in experimental animals and in certain patients with Cushing's syndrome was not noted in this study. Repeated electrocardiograms and teleroentgenograms were unaltered.

Consistent small increases in "resting"[2] blood pressure were observed in all 5 cases during the first week of DCA administration. Blood pressure levels remained elevated in 3 patients until the drug was discontinued; but in one subject receiving 10 mg per day and in the subject receiving 20 mg per day the blood pressure returned to control values after a transitory rise (Fig. 1). The evidences of temporary salt and water retention were present in all, but disappeared whether or not blood pressure changes were sustained. The serum volume, determined in a second patient who exhibited a persistent increase in arterial tension, was unchanged before and at the close of DCA therapy.

Following the cessation of steroid injection, the serum potassium concentration returned to control values and the carbon dioxide content and chloride concentrations reversed their trends. There was no evidence of abnormal sodium, chloride or water loss in

these patients with a fixed fluid intake except during the second week of DCA treatment, at which time the initial salt and water retention was disappearing. This is in contrast to the excess diuresis and sodium loss previously reported in a non-hypertensive.[4]

Discussion. Although the rise in "resting" blood pressure, observed within a few days after the sustained administration of DCA in hypertensive subjects, has been confirmed, the increase may be temporary or may continue to be apparent throughout the period of daily drug administration. Salt and water retention does not appear to be the sole determining factor, as it is transitory even in the presence of persistent increases in blood pressure above baseline values.

TABLE I.

Serum electrolyte changes induced by DCA administration in 5 hypertensive patients.

Serum determination (meq/liter)	Start of DCA	End of DCA
CO_2 Content	26.6	29.7
	25.0	30.7
	29.7	31.2
	25.6	30.4
	33.0	38.8
Chloride	105.8	102.3
	104.6	99.6
	102.3	99.2
	104.0	99.4
	93.4	88.4
Sodium	136.7	140.1
	140.4	138.2
	137.9	139.5
	136.8	139.5
	140.4	140.2
Potassium	4.2	3.4
	4.3	3.2
	3.3	2.8
	4.8	2.9
	3.3	2.3

The fact that the pressor response to DCA is a matter of days rather than of hours suggests that a direct humoral effect is not responsible. This is further confirmed in preliminary studies following the single intravenous injection of desoxycorticosterone glucoside (Ciba) to hypertensive patients in doses from 5 to 30 mg; no significant change in blood pressure was observed with this preparation in 3 subjects over a 30-minute period of sphygmomanometric readings taken every minute (excluding the transitory elevation which may arise immediately after the introduction of the needle).[5]

Summary. The daily injection of DCA, in 5 patients with uncomplicated hypertensive vascular disease for periods of 30 days or more, produced a transitory rise in "resting" blood pressure in all instances, a sustained increase during the period of drug administration in but 3 of the 5 subjects.

The continued injection of DCA was associated with a progressive drop in serum potassium concentration, an increase in carbon dioxide content and a fall in chloride concentration. Salt and water retention, on the other hand, was observed only during the first 7-10 days of treatment, with reversal to control levels thereafter.

The pressor action of DCA does not appear to be due to a direct humoral mechanism dependent on its concentration in the circulation.

[4] Zierler, K. L., and Lilienthal, J. L., Jr., *Am. J. Med.*, 1948, **4**, 186.

[5] Goldman, M. L., and Schroeder, H. A., *Science*, 1948, **107**, 272.

16388

Method for Determining Amino Acid Adequacy of Liquid Protein Hydrolysates in Rats.

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Experiments designed to measure the nutritive value of hydrolysates given parenterally[1] and orally[2] for growth of young rats have not met with marked success. The method of depletion of adult rats described by Cannon, *et al.*[3,4,5] appeared to offer certain advantages. Adult protein-depleted rats will voluntarily consume large volumes of liquid food,[6] have a great avidity for nitrogenous foods, and typify the clinical condition wherein the use of intravenous protein hydrolysates is indicated.

Method. Young adult male rats, 140-220 g, were placed on the following depletion diet for 12 days: sucrose 83, salt mixture No. 1 (U.S.P.) 4, agar 1.4, primex 4.2, corn oil 4.2, cod liver oil 1.4, choline chloride 0.15, and inositol 0.14 g; thiamine hydrochloride 0.6, riboflavin 1.2, pyridoxine hydrochloride 0.6, calcium pantothenate 5.0, nicotinic acid 3.7, mixed tocopherols 2.5 and ascorbic acid 14 mg per 100 g diet. Average weight loss was 24% of body weight, range 21-28%. The non-protein diet was fed *ad libitum*. The 5% hydrolysates, or amino acid solutions, were fed in drinking tubes attached to the cages. Water was not generally supplied to groups fed the liquid hydrolysates.

To avoid the somewhat variable response during the first 3 days of the 12-day assay period the following practice was adopted. At the end of the 12-day depletion the rats are offered a standard partial acid hydrolysate of fibrin[7] *ad libitum* for 3 days. During this trial drinking period the rats rapidly increase their consumption to 40 cc or more per day. The rats are then returned to the non-protein diet for 3 days for redepletion. Rats which do not respond well can be discarded here. When the liquid hydrolysate is again offered to rats prepared in this way, they regularly consume 40 cc or more per day for 12 days.

The rats are weighed before the initial feeding at the beginning of the 12-day assay period, and again 24 hours after the last feeding. In this way the possibility of weighing just after the animal has consumed a large volume of solution is avoided. Because no water is offered more concentrated hydrolysates are diluted to about 0.65% nitrogen before feeding. This level of nitrogen represents about 5% hydrolysate solids.

In *ad libitum* feeding trials with acid hydrolysates of fibrin rats consumed an average of 50-60 cc daily, equal to 0.32-0.4 g N. Separate experiments indicated that the nitrogen efficiency ratio (ratio of weight gain to N intake) is at a peak at 0.24 g N per day. This level provides the equivalent of 1.5 g average protein per day. In the present study both *ad libitum* and restricted feeding methods are reported. Continuing studies have been carried out chiefly with restricted feeding—the depleted rats readily consume the allotment of about 35 cc of 5% hydrolysate equal to 0.24 g N in 10-20 hours.

Application. Ad libitum liquid hydrolysate feeding was compared with feeding the dried hydrolysate added to the diet at a level to supply 2% N. Comparison was also made with intact fibrin at a level which supplied

1 Horwitz, A., Sachar, L. A., and Elman, R., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 118.

2 Mueller, A. J., *Ind. Eng. Chem. Anal. Ed.*, 1945, **17**, 639.

3 Wissler, R. W., Woolridge, R. L., Steffee, R. H., and Cannon, P. R., *J. Immunol.*, 1946, **52**, 267.

4 Frazier, L. E., Wissler, R. W., Steffee, C. H., Woolridge, R. L., and Cannon, P. R., *J. Nutrition*, 1947, **33**, 65.

5 Wissler, R. W., Steffee, C. H., Woolridge, R. L., Benditt, E. P., and Cannon, P. R., *J. Am. Diet. Assn.*, 1947, **23**, 841.

6 Adolph, E. F., *Fed. Proc.*, 1947, **6**, 67.

7 Frost, D. V., Heinsen, J., and Olsen, R. T., *Arch. Biochem.*, 1946, **10**, 215.

TABLE I.

Comparison of Different Methods of Feeding Protein Hydrolysate. Comparison with Whole Proteins.

Supplement	How supplied	No. of rats	Intake of allotted N, %	Weight gain Range, g	Weight gain Avg. g
Casein	Separate from diet: 0.24 g N/day	5	96	27-52	44
Fibrin	Separate from diet:				
	0.24 g N/day	4	100	58-60	59
	0.32 " "	5	99	53-74	68
	At 1.9% N in diet:	6	—	53-80	70
5% Fibrin† Hydrolysate	35 cc = 0.24 g N/day	6	100	44-55	49
	47 " 0.32 " "	5	100	47-65	56
	Ad libitum:	12	—	44-77	61
Hydrolysate dried	At 2% N in diet:	5	—	49-73	65

* Aminosol is the trade name for the intravenous partial acid hydrolysate of fibrin used in these studies.

1.9% N in the diet.

Further experiments were carried out wherein powdered, purified fibrin and casein were fed as separate supplements at levels of 0.24 and 0.32 g N per day for comparison with a 5% partial acid hydrolysate of fibrin fed at similar levels of nitrogen. The dry proteins were fed in small cups attached to

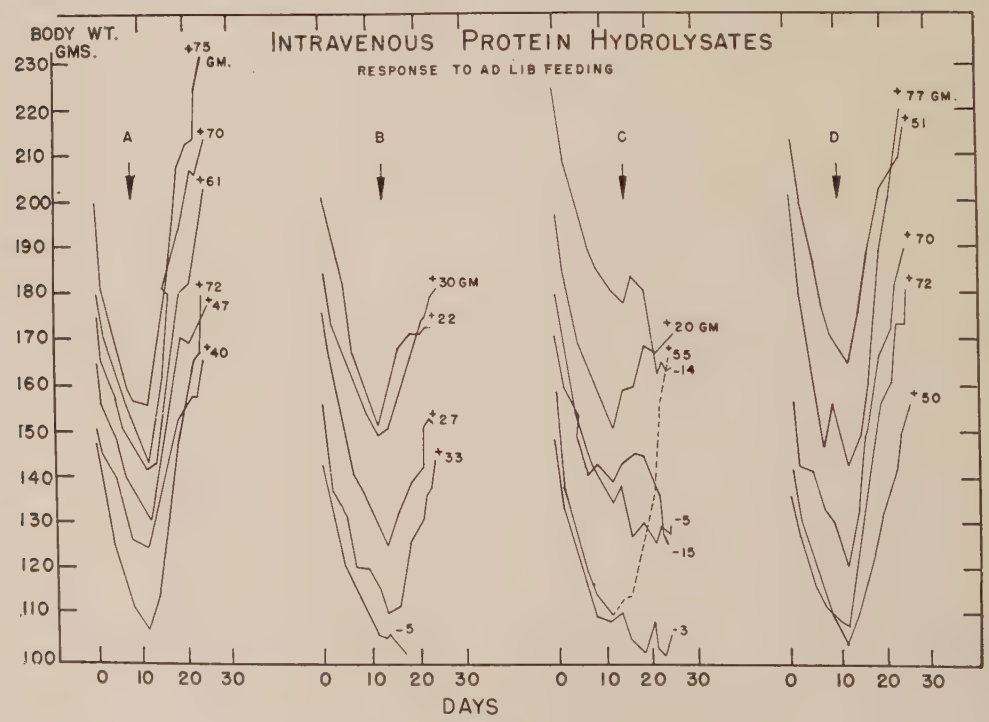

FIG. 1.

Weight changes of individual rats in 4 groups during depletion on non-protein diet and during repletion following *ad. lib* feeding of 4 different protein hydrolysates. The marked weight gain of one of the rats on hydrolysate C is shown with a dotted line because it is quite different from the responses of other rats on this hydrolysate. The 12-day weight gains or losses of the individual rats are shown to the right of each curve.

TABLE II.
Essential Amino Acid Analysis of Four Intravenous Protein Hydrolysates and of Whole Egg (Calculated to 16% N).

	A %	B %	C %	D %	Defatted whole egg %
Isoleucine	4.6	4.9	3.6	6.3	5.7
Leucine	6.5	6.9	9.3	9.5	9.1
Valine	4.8	5.9	7.7	7.4	6.4
Threonine	6.7	3.3	7.1	4.4	4.9
Methionine	2.6	3.0	1.1	2.7	3.3
Cystine	2.0	1.2	1.7	1.4	2.4
Phenylalanine	3.4	2.2	3.5	4.7	5.5
Tryptophan	1.0	0.4	0.4	0.9	1.5
Lysine	8.4	6.8	7.8	7.6	6.0
Histidine	2.3	2.3	2.2	2.5	2.5
Arginine	5.7	3.2	3.5	2.9	6.4

Cystine was determined chemically by the method of Folin and Marenzi,[12] methionine by the method of White,[11] and tryptophan by the method of Graham *et al.*[10] All other amino acids were determined microbiologically by the method of Stokes *et al.*[9]

the cage walls by metal clips. Water was offered *ad libitum* to these groups. The rats accepted these particular dried proteins with avidity throughout; however, subsequent trials with other proteins have not been generally as successful. A summary of the above experiments is shown in Table I.

Protein hydrolysates designated A, B, C and D made from different proteins and known to differ in amino acid value were assayed. The hydrolysates were all made to contain .65% nitrogen before feeding. Both the protein-free diet and the hydrolysates were fed *ad libitum* to all groups. The depletion and growth responses are shown in Fig. 1.

The 4 hydrolysates were then subjected to analysis for the essential amino acids. As a reference protein of known high biological value defatted whole egg was analyzed simultaneously, as suggested by Block and Mitchell.[8] The analytical results and references to the methods used are shown in Table II. The limiting amino acids for hydrolysate B appear to be tryptophan and phenylalanine, and for hydrolysate C, tryptophan and methionine. No marked deficiencies appear in hydrolysates A and D. The magnitude of the repletion response appeared unrelated to the original weights of the rats over a rather wide range.

Amino Acid Mixtures in Solution. A mixture of 16 amino acids* patterned after casein (Ration A of Frazier, *et al.*)[4] was found to be somewhat less than completely soluble at a level of 5% solids in water. Adjustment of the pH to 5.5 or to 7.5 did not correct this problem. The solution used for feeding was pH 5.5. The solution was diluted to 4% solids and an equal amount of dextrose was added. On heating the amino acids went into solution; however, slight crystallization of tyrosine occurred again on cooling. Cysteine hydrochloride was added at a level of 0.6% of the amino acid solids to correct partially the known deficiency of total sulfur amino acids. This amino acid mixture was designated Amino Acid Solution A_1 and the composition is shown in Table III.

A preliminary comparison was made of the growth response of standard depleted rats to powdered casein and to the Amino Acid Solution A_1. The casein was fed in cups attached to the sides of the cages and the rats in this

8 Mitchell, H. H., and Block, R. J., *J. Biol. Chem.*, 1946, **163**, 599.

9 Stokes, J. L., Gunness, M., Dwyer, J. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

10 Graham, C. E., Smith, E. P., Hier, S. W., and Klein, D., *J. Biol. Chem.*, 1947, **168**, 711.

11 White, W. F., *J. Biol. Chem.*, 1945, **158**, 535.

12 Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, 1929, **83**, 109; 1934, **106**, 311.

* Crystalline amino acids were obtained chiefly from Merck; also from Dow, Winthrop, Pfanstiehl and Monsanto. Purity was established by chemical or microbiological assay before use.

group were given water *ad libitum*. Both supplements were offered in amounts to supply 0.24 g N per rat day. Of 6 rats in this group, one failed to take the dry casein at all and was discarded, while the remaining 5 gained 27, 40, 48, 52 and 52 g.

Two rats, similarly prepared, received 48 cc per day of Solution A_1 and gained 52 and 55 g. The rats consumed 93% of their liquid allotments. This response is especially significant in view of the fact that the synthetic form of certain of the amino acids used, *i.e.* valine, threonine, isoleucine and phenylalanine have been shown to be incompletely used by the growing rat, the D-isomer being partly or completely rejected.[13] Further work with complete hydrolysates of fibrin fed similarly has indicated that responses of a very high order are regularly obtained with solutions containing only amino acids.

TABLE III.
Composition of Amino Acid Mixtures Fed in Solution.

	Sol. A-1 contained 4% of mixture, g/100 g	Sol. A-4 contained 5% of mixture, g/100 g
l-Arginine HCl	4.21	4.0
l-Histidine HCl	2.87	3.0
dl-Isoleucine	11.05	11.0
l-Leucine	10.29	10.0
l-Lysine HCl	8.05	8.0
dl-Methionine	2.98	5.0
dl-Phenylalanine	4.42	9.0
dl-Threonine	6.63	6.5
dl-Tryptophan	1.53	1.5
dl-Valine	11.9	12.0
dl-Alanine	4.76	6.0
dl-Aspartic acid	5.36	5.0
l-Cystine	0.31	—
l-Glutamic acid	20.16	14.0
Glycine	0.42	5.0
l-Tyrosine	5.44	—
	100.38	100.0

l-Cysteine hydrochloride monohydrate was added to Solution A-1 at a level equal to 0.6% of the amino acid solids.

An attempt was made to compensate for the insolubility of certain of the amino acids and to prepare a mixture of amino acids of equal nutritive value to amino acid solution A_1, but one which would be soluble to the extent of 5% in water. Solution A_4 (Table III) was developed along this line and was made up for feeding with 5% dextrose. The pH was 4.7. In order to demonstrate the effect of complete omission of one of the essential amino acids, mixture A_4 minus isoleucine was made up similarly.

The effect of feeding Solution A_4 and Solution A_4 minus isoleucine to standard depleted rats is shown in Fig. 2. Because they received no water other than that in the amino acid solution the rats were forced to take some of the incomplete mixture if only to satisfy thirst; however, they showed an actual aversion for the solution and drank an average of only about 2 cc per day. The response of the 6 rats to Solution A_4 was unusually erratic and only 70% average allotment was consumed. In repeat experiments responses of about 60 g have been obtained with much less variation than that shown by the present group. A gradual decrease in intake of this type of solution has been noted and suggests a possible inhibitory effect of the *D*-amino acids present.

Discussion. The data at hand suggest that adequacy of the 9 amino acids described by Rose[13] and established in the rat repletion assay by Frazier, *et al.*[4] is the most important single criterion which must be met if a hydrolysate is to be of high nutritive value. On failure of a hydrolysate by the rat repletion technic, one would look first for limitations in the essential amino acid composition, as has been done in the above experiments. Such questions as palatability to the rat and the role played by the so-called dispensable amino acids require individual study.

An advantage of the method is the ability to feed the liquid supplement accurately and rapidly. No wastage of the nitrogen component occurs and measurement of any liquid not consumed is convenient. The nutritive adequacy of the nitrogen component determines the appetite of the animal for the non-nitrogenous part of the diet. The practice of rationing only the nitrogen component at a level of 240 mg N per day appears to provide a fairly critical assay with a minimum of handling.

Summary. The 12-day weight response of

[13] Rose, W. C., *Physiol. Rev.*, 1938, **18**, 109.

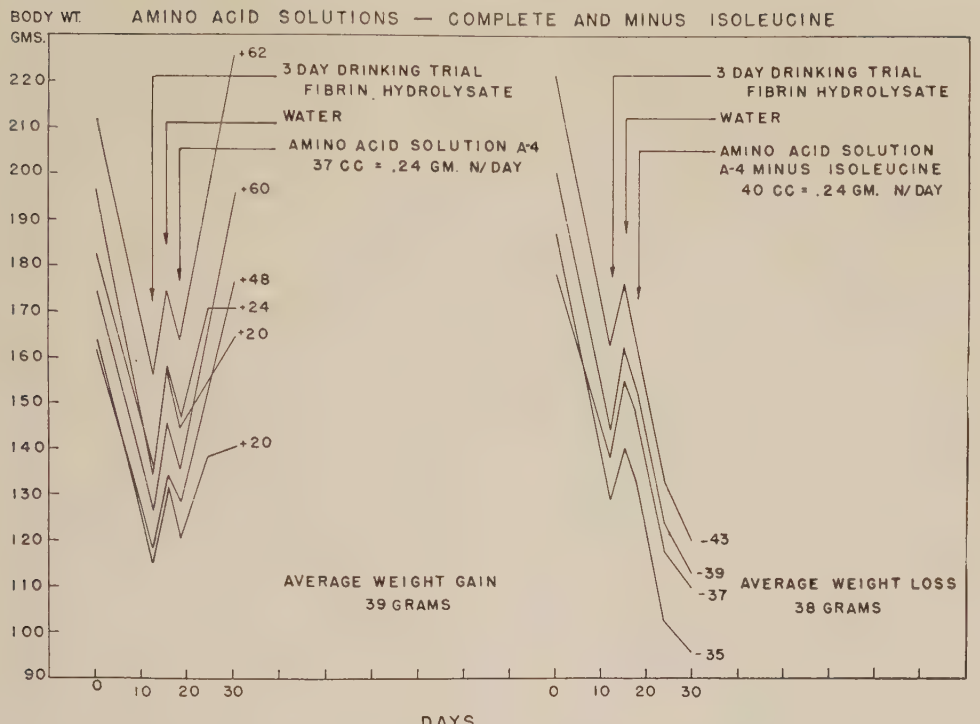

FIG. 2.

Weight changes of individual rats during 12-day depletion, 3-day orientation to liquid feeding, 3-day redepletion and final assay periods. Assay of a 5% amino acid solution patterned after the amino acid composition of casein (corrected for insolubility of tyrosine, cystine, and glutamic acid) versus the similar mixture minus isoleucine.

young adult protein-depleted rats to 5% protein hydrolysates fed as the sole source of amino acid nitrogen provides a basis for rapid assay. The response to liquid hydrolysate feeding approached that of dried hydrolysate or whole protein added to the diet. Pure amino acid solutions of good nutritive composition are readily accepted and support good weight recovery. The effect of the *D*-amino acids in such mixtures requires clarification.

Acknowledgement is made to Eleanor Willerton for microbiological amino acid assays and to E. O. Krueger for the chemical assays. We wish also to thank Dr. Paul R. Cannon for his helpful counsel in these studies.

16389

Enzyme Studies on Human Blood. II. The Substrate in the Fibrinogen-Thrombin Reaction.*

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Divergent views exist in respect to the influence of fibrinogen concentration on the fibrinogen-thrombin reaction. An optimal range of substrate concentration for the velocity of the fibrinogen-thrombin reaction has been reported.[1,2,3,4] In this communica-

* Fibrinogen fractions were prepared from dried normal human plasma which had been processed from blood obtained from volunteer donors enrolled by the American Red Cross.

tion, the results of 51 consecutive experiments are presented, confirming the existence of an optimum concentration for the substrate. The relationship between this optimum concentration and the nature of the fibrinogen preparation has been investigated.

Methods and Material. Fibrinogen preparations (Fraction I) were obtained from dried human plasma by the low temperature-ethanol procedure, Method 6, of the Department of Physical Chemistry, Harvard Medical School.[5] The precipitate was formed in a low temperature bath at –2.5 to –3.0°C and separated in a refrigerated centrifuge (International PR 1). Subfractions of Fraction I were also prepared by low salt, low temperature procedures. All protein precipitates were lyophilized at an initial temperature of –70°C, and at less than 0.1 mm pressure. The dry powder was stored in a vacuum desiccator. One or 2% solutions of the fibrinogen fractions in a buffer were prepared on the days of the experiment.

The thrombin employed, prior to its expiration date, was Lederle's "Hemostatic Globulin."[6] The activity of this product was quite constant, day to day and vial to vial, as determined by experiments with a standard fibrinogen preparation. For the measurement of clotting times a 1:10 dilution of the material with the appropriate buffer was prepared. This solution retained its potency at room temperature for at least 6 hours. For the estimation of thrombin-clottable protein, a 1:60 dilution of dialyzed thrombin was prepared.

All reagents were dissolved or diluted in either the Michaelis veronal buffer as modified by Owren,[3] pH 7.3, Γ/2 (ionic strength) 0.154, or a citrate-phosphate buffer, pH 7.2, Γ/2 0.129. The latter consists of 250 cc 1/15 M Sorensen buffer, pH 7.1, and 75 cc 0.1 M sodium citrate in one liter solution. The pH was determined with a glass electrode electrometer at 25°C.

The fibrinogen was estimated by two methods. In the thrombin technic, 1 cc of approximately 1% protein solution was added to 7 cc of buffer and 2 cc of 1:60 dialyzed thrombin and allowed to stand for 3 hours; in the heat technic, an approximately 0.1% protein solution was heated at 54-56°C in a water bath for 5 minutes. After centrifugation the macro Folin-Ciocalteu[7] tyrosine equivalents were determined, of the supernatant fluid, the untreated protein solution, and the thrombin reagent. As an additional correction factor, acid soluble "tyrosine" values were obtained on various fibrinogen preparations by the micro-technic. This colorimetric method as employed in this laboratory has an approximate average sensitivity of 0.003 mg tyrosine per 1% T for the macro-technic, and 0.001 mg per 1% T for the micro-technic on the Coleman Junior Spectrophotometer.

The clotting times were measured as follows: The substrate (0.8 cc) was pipetted into a test tube (*i.d.*, 1 cm) and equilibrated in a 37.5°C water bath. The stop watch, with a hidden face, was started at the moment 0.2 cc of the thrombin was blown in. In this study, the criteria for the end-point were: the characteristic gel formation, or the appearance of clumped fibrin granules or threads. The latter appeared in the test tube simultaneously with complete disappearance of any opacity in the solution. The latter criterion was necessary when experimenting with dilute fibrinogen solutions.

Results. The data from two experiments on a fibrinogen fraction (Run 251) are presented in Fig. 1. They are typical of the results obtained in each of the 51 experiments on 34 preparations. The concentration-clotting time curve is U-shaped and the clotting times are

[1] Wöhlisch, E., Diebold, W., and Kiderlin, O., *Arch. ges. Physiol.*, 1936, **237**, 599.

[2] Wöhlisch, E., and Neugschwender, A., *Biochem.*, 1937, **292**, 196.

[3] Owren, P. A., *Acta med. Scand.*, 1947, **128**, (Supl. 194), 1.

[4] Ferry, J. D., and Morrison, P. R., *J. Am. Chem. Soc.*, 1947, **69**, 388.

[5] Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.

[6] Parfentjev, I. A., *Am. J. Med. Sci.*, 1941, **202**, 578.

[7] Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

higher for a given fibrinogen preparation in the citrate-phosphate buffer than in the Michaelis veronal buffer. In Table I the results from all experiments are summarized and grouped according to the range of fibrinogen purity and the buffer used.

Discussion. Our experiments confirm the findings of Wöhlisch *et al.*[1,2] and others[3,4] that there is an optimum substrate concentration for the thrombin-fibrinogen reaction velocity. In any comparison of the range of the optimum concentration reported here and by Ferry and Morrison,[4] with those of other investigators, it must be recognized that fibrinogen fractions, prepared by the low temperature-ethanol procedure, are not "pure" as shown by quantitative data. Ferry and Morrison[4] found the minimum concentration to

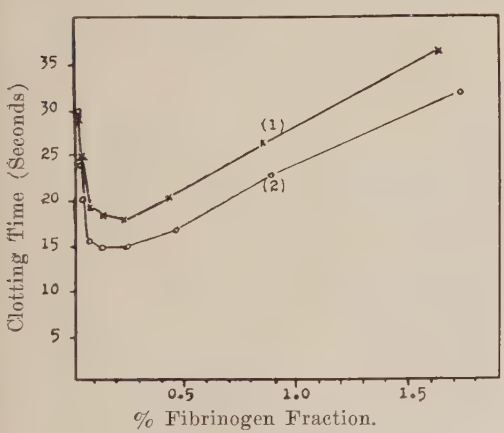

FIG. 1.

Relation of fibrinogen fraction concentration to thrombin clotting time: fibrinogen in fraction, 78.2% (thrombin technic) and 77.2% (heat technic). (1) Citrate-phosphate buffer, Γ/2, 0.129; pH, 7.2. (2) Michaelis veronal buffer, Γ/2, 0.154; pH, 7.3.

be approximately 0.1-0.2% in an experiment with a fibrinogen fraction of 74% purity. Other investigators tested either plasma or fibrinogen prepared by the classical ammonium sulfate principle and of a purity sometimes not given. Thus, Wöhlisch[1,2] demonstrated an optimum fibrinogen concentration, first at 0.2-0.4% and later at 0.05%. Within the limits of our experimental conditions, the over-all optimum range of the substrate was approximately between 0.1-0.3%, calculated as fibrinogen.

TABLE I.

Thrombin Clotting Time of Fibrinogen Solutions at Various Concentrations: A Summary of 51 Experiments.

Fibrinogen purity %	No. of Prep.	No. of Exp.	Buffer	Clotting time* in seconds ± S.D. % fibrinogen fraction in substrate 1.0	0.5	0.25	0.125	0.062	0.031	0.016
60-69	12	18	M.V.	17.2 ± 2.1	13.9 ± 1.4	13.0 ± 1.0	13.6 ± 0.2	16.5 ± 1.0	24.3 ± 2.8	46.4 ± 8.3
		2	C.P.	27.7 ± 0.9	19.5 ± 2.9	17.4 ± 0.8	17.7 ± 0.2	19.2 ± 0.4	23.4 ± 0.8	31.5 ± 2.1
70-79	19	19	M.V.	20.6 ± 3.4	15.4 ± 2.1	13.4 ± 1.2	13.5 ± 0.9	15.4 ± 1.6	19.9 ± 3.0	31.3 ± 8.4
		1	C.P.	35.0	25.0	20.4	18.6	19.2	22.0	30.8
80-84	3	3	M.V.	23.6 ± 3.4	16.3 ± 1.5	14.6 ± 0.3	14.2 ± 1.7	15.3 ± 2.3	21.1 ± 3.1	38.7 ± 9.0
		8	C.P.	26.5 ± 2.3	20.1 ± 1.1	17.7 ± 0.6	17.5 ± 0.7	18.4 ± 1.1	22.1 ± 1.7	31.0 ± 3.1

* 0.2 cc thrombin added to 0.8 cc of substrate, 37.5°C.
M.V., Michaelis Veronal, pH 7.3, Γ/2 0.154.
C.P., Citrate-Phosphate, pH 7.2, Γ/2 0.129.

In general, the level of the clotting times at concentrations of fibrinogen fraction over 0.25% becomes elevated as the purity of the preparation increases. This elevation in the reaction time cannot be accounted for by the absolute increase in the concentration of fibrinogen. A possible explanation is that further purification of Fraction I resulted in preparations containing relatively more anti-coagulant and/or less pro-coagulant factors. The minor components of Fraction I are alpha-, beta-, gamma-globulins and albumin.[5] An antihemophilic "globulin"[8] and an active proteolytic enzyme[9] have also been reported in this fraction. Thus far only the anti-hemophilic globulin has been reported as having a direct influence on the coagulation. The influence of the other minor components of Fraction I upon the clotting time is being studied in our laboratory.

Summary. Fifty-one experiments on 34 fibrinogen fractions prepared by the low temperature-ethanol principle confirm the reports of those investigators who observed an optimum concentration of substrate for the thrombin-fibrinogen reaction. The results also demonstrate that the level and range of this optimum concentration are determined by the nature of the fibrinogen preparation.

The valuable technical assistance of Mary Beth Everhart is gratefully acknowledged.

8 Taylor, F. H. L., Davidson, C. S., Tagnon, H. J., Adams, M. A., MacDonald, A. H., and Minot, G. R., *J. Clin. Invest.*, 1945, **24**, 698.

9 Shinowara, G. Y., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 456.

16390

Nutrition of the Mouse. VII. Lactation Performance of Four Strains Maintained on Stock Rations.*

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Highly purified diets adequate to support growth of rats and mice are in common use today. The adequacy of such rations for reproduction and lactation (especially under conditions reducing the opportunity for coprophagy) is still open to some doubt.[1–6] The completeness of a diet for reproduction and lactation can be judged by several criteria, among which the weaning weights of the young and the weight changes of the female during the lactation period have been frequently employed. Too often, however, purified diets have been compared, not with excellent, but with mediocre stock rations. In order to indicate the standards which a synthetic diet must meet, we wish to report our observations with mice of 4 highly inbred strains maintained in our breeding colony on various stock diets.

Methods. The procedure for maintaining the breeding colony has been described previously (Fenton and Cowgill).[6] A commercial ra-

* Supported by grants from the Anna Fuller Fund, and the American Cancer Society, on recommendation of the Committee on Growth of the National Research Council.

1 Rogers, L. K., McElroy, L. W., and Cowgill, G. R., *Science*, 1942, **95**, 203.

2 Foster, C., Jones, J. H., Dorfman, F., and Kobler, R. S., *J. Nutrition*, 1943, **25**, 161.

3 Cerecedo, L. R., and Vinson, L. J., *Arch. Biochem.*, 1944, **5**, 157.

4 Cerecedo, L. R., and Mirone, L., *Arch. Biochem.*, 1947, **12**, 154.

5 Mirone, L., and Cerecedo, L. R., *Arch. Biochem.*, 1947, **15**, 324.

6 Fenton, P. F., and Cowgill, G. R., *J. Nutrition*, 1947, **33**, 703.

TABLE I.
Weight Changes of Mothers and Young During 3-Week Lactation Period.

Group	Strain	Generation	Diet	No. of litters	Weeks after parturition: Wt of mothers, g 0	1	2	3	Avg wt of young, g 0	1	2	3
I	C57	P	L*	23	25.6	28.8	29.5	28.3	1.2	3.2	5.9	7.3
	A	P	L	18	25.0	25.6	26.3	24.9	1.2	3.2	5.4	6.8
II	C57	P	L	58	27.7	31.1	32.2	29.8	1.3	3.6	6.5	8.7
	A	P	L	41	30.0	30.4	29.5	28.6	1.3	3.6	6.2	7.9
	C3H	P	L	10	30.2	32.9	33.8	31.5	1.5	4.6	7.0	9.6
III	C57	F_1	L	42	27.4	30.6	31.3	29.6	1.3	3.9	6.8	9.4
	A	F_1	L	44	28.9	30.6	29.9	28.9	1.3	3.7	6.4	8.5
	C3H	F_1	L	12	34.3	33.4	36.3	32.0	1.5	4.5	7.6	9.6
IV	C57, A	F_1	153	10	29.0	31.5	33.0	28.0	1.3	4.7	8.7	11.1
V	C57	F_1-F_2	153	34	24.6	27.0	28.5	25.2	1.3	4.1	7.7	10.6
	A	F_1-F_2	153	24	26.6	27.3	27.8	26.8	1.3	4.3	8.4	10.9
VI	C57	F_1-F_2	153	18	26.7	29.5	31.4	27.4	1.3	4.1	7.9	11.1
	A	F_1-F_2	153	13	29.0	29.6	30.0	28.7	1.4	4.5	9.1	11.8
VII	I	P	153	4	22.4	23.6	22.8	21.7	1.2	4.1	6.1	8.4

* Purina Laboratory Chow.

tion† and a stock diet (No. 153) prepared in the laboratory were employed. The latter was fed only during the lactation period wherever indicated in Table I. Diet 153 had the following percentage composition: dried whole milk (Klim) 50, Ruffex 2, Sure's[7] salts 5, Wilson's liver substance 3, dried yeast 10, corn oil 10, casein 10, and dextrin 10. The corn oil used in this ration was the same preparation (containing the fat-soluble vitamins) which we have used in some of our synthetic diets. Food and water were supplied *ad libitum*. Although in nearly all cases the number of young was reduced to 8 at birth and to 6 at the end of the first week of the lactation period, in some instances toward the end of this study some litters were raised to weaning without such reduction in the number of young.

The data reported here were secured on a total of 351 litters, with 1,762 young surviving the 3-week lactation period.

Results. All pertinent observations are summarized in Table I. The parent (P) generation consisted of animals which we received at the beginning of our work.‡ They are reported in the table under 2 separate headings (Groups I and II). Group I consists of data accumulated during the early phase of our experiments when considerable experimentation was carried out to determine the best method of housing and handling the breeding colony and lactating females with litters. Group II includes all data obtained with this same group of mice after the methods of maintenance had been standardized. These methods have not been significantly changed since. Group IV consists of data secured from both C57 and A strain litters. They are grouped together in the table because they were few in number and constituted a pilot experiment to determine whether or not diet 153 could be considered to be superior to the commercial feed previously used. When this proved to be the case, the entire stock breeding colony was changed over to the new ration. It should not be thought that the animals in Group IV were hybrids of the C57 and A strains. Diet 153 was fed to the breeding females from parturition to the end of the 3 week lactation period; at all other times these animals were supplied the commercial feed. The data in Groups V and VI were obtained from the same series of animals, Group V showing the weight changes with the first litters, Group VI indicating the results with all subsequent litters of the animals in Group V. Group VII shows the results obtained with females of the I strain, with which we have just recently begun to work. A few C3H litters have been reared

† Purina Laboratory Chow.

[7] Sure, B., *J. Nutrition*, 1943, **26**, 275.

‡ Obtained through the courtesy of Dr. L. C. Strong and Dr. A. Gorbman, Department of Anatomy, Yale University.

on diet 153. Although they too showed improvement, the data are not included in the table since there were too few to warrant the expenditure of table space.

Discussion. Two facts stand out in the data presented. First of all it is clear (and was to be expected) that improvements in the method of handling the mouse colony resulted in improved lactation performance, as shown by the contrast in weights of the young in Groups I and II. It is also clear that improvement in the diet fed during the lactation period resulted in improved weaning weights of the young. Of course, one cannot overlook the possibility that maintenance of mice under the carefully controlled conditions of our colony would result in some improvement of lactational performance, even without alteration in the dietary regime. Our own experience with purified diets[6] has convinced us of the inadequacy of such diets when compared with an excellent stock ration. Whether this is due to an imbalance of the components or to the absence of unidentified factors remains to be determined.

Summary and Conclusions. 1. Lactation performance, as judged by weight changes of the mothers and weaning weights of the offspring, has been studied with four highly inbred strains of mice. The data reported were secured from 351 litters and a total of 1,762 offspring successfully weaned. 2. Definite improvement in the weaning weights of the young was secured. Three factors probably played a role: a) improvement in the method of handling the breeding colony, b) maintenance of the breeding colony under excellent nutritional conditions through several generations, and c) improvement in the diet fed during the lactation period.

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Resistance of Guinea Pigs to Action of Alloxan.*

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Since the demonstration by Dunn and his collaborators[1] that alloxan produces necrosis of the beta cells of the islets of Langerhans with the development of persistent diabetes in rabbits, a number of investigators have reported similar effects following its administration to a variety of animals.[2,3,4,5]

The dosage of alloxan required for production of the diabetic state varies widely with animal species. Goldner[6] gives the following species gradation according to decreasing sensitivity to alloxan: dogs, monkeys, pigeons and cats, rabbits, rats. Shultz and Duke[7] found rabbits before the 9th day of life to be resistant to doses of alloxan which produced diabetes in older animals. Goldner[6] observed that guinea pigs are resistant to alloxan, and showed pancreatic lesions only if death ensued within 24 hours after the injection. He gave no data on blood sugar levels or urinary sugar excretion. Saviano and De Franciscis[8] gave alloxan in doses of 50-450 mg/kg to guinea pigs with the production, generally, of an intense hypoglycemia which persisted and

* Aided by a grant from the Diabetic Research Foundation of Portland, Oregon.

1 Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet,* 1943, **1**, 484.

2 Gomori, G., and Goldner, M. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 287.

3 Banerjee, S., *Lancet,* 1944, **2**, 658.

4 Carrasco-Formiguera, R., *J. Lab. and Clin. Med.,* 1944, **29**, 510.

5 Ruben, J. A., and Yardumian, K. W., *Science,* 1946, **103**, 220.

6 Goldner, M. G., *Bull. N. Y. Acad. Med.,* 1945, **21**, 44.

7 Shultz, C. S., and Duke, J. R., *Bull. Johns Hopkins Hosp.,* 1948, **82**, 20.

8 Saviano, M., and De Franciscis, P., *Boll. soc. ital. biol. sper.,* 1947, **23**, 307.

TABLE I.
Effect of Alloxan on Urine Sugar in the Guinea Pig.

Animal No.	Wt, g	Alloxan, mg/kg	Route of administration	Glycosuria
		Normal Animals		
1	260 fasting	100	subcutaneous	—
1	264 "	100	"	—
1	278 "	175	"	—
1	397 nonfasting	600	"	—
2	276 fasting	125	"	—
2	279 "	300	"	—
3	273 "	175	"	—
3	296 "	300	"	—
3	388 nonfasting	600	"	—
4	490 "	600	intraperitoneal	Animal died without sample
		Scorbutic Animals		
5	336 nonfasting	200	subcutaneous	—
5	336 "	600	"	—

caused death as late as 11 days after alloxan administration.

Stoll[9] reported that the effects of ninhydrin on islet cells of the pancreas are the same in the rat and guinea pig and that this effect is similar to the effect obtained by alloxan. No specific data was given concerning the effect of alloxan on the islet cells in guinea pigs and no mention was made concerning its effect on blood or urine sugar in these animals.

In conformity with observations of the above workers, the writers have found that guinea pigs are exceptionally resistant to the toxic effects of alloxan. Animals maintained under a variety of conditions have failed to respond by appreciable hyperglycemia or glycosuria to doses of alloxan varying from 100 to 1,000 mg/kg administered sub-

[9] Stoll, W., *Z. Naturforsch.*, 1946, **1**, 592.

TABLE II.
Effect of Alloxan on Blood Sugar of Normal Guinea Pigs.

Animal No.	Wt, g	Alloxan mg/kg	Route of administration	Nonfasting blood sugar, mg %
1	621 nonfasting	1000	subcutaneous	130
2	365 fasting	1000	"	170
3	472 "	1000	intraperitoneal	90
4	— nonfasting	600	"	93
5	480 "	300	"	123
6	480 "	300	"	123
7	480 "	300	"	123
8	450 "	300	"	107
9	540 "	300	"	113
10	480 "	300	"	90
11	480 "	300	"	102
12	405 "	300	"	113
13	465 "	300	"	141
14	525 "	300	"	135
15	465 "	300	"	147
16	600 "	350	intracardial	(died)
17	400 "	350	"	"
18	200 "	350	"	"
19	200 "	350	"	"
20	200 "	350	"	"
21	200 "	350	"	"
22	200 "	350	"	136
23	411 "	150	"	119
24	556 "	150	"	102
25	290 "	150	"	113
26	321 "	150	"	126

TABLE III.
Effect of Alloxan on the Blood Sugar of Scorbutic Guinea Pigs.

Animal No.	Wt. g	Alloxan mg/kg	Description	Nonfasting blood sugar, mg %
1	400 nonfasting	1000	scorbutic	147
2	400 "	1000	"	(died)
3	405 fasting	1000	"	113
4	475 "	1000	" (plus chloretone)	90
5	480 "	1000	" " "	124
6	430 "	1000	" " "*	203
7	375 "	1000	" " "	90

* Animal dead when blood was drawn.

cutaneously, intraperitoneally, and intracardially.

Experimental procedure. Normal animals, scorbutic animals, and scorbutic animals which had received 80 mg of chloretone by mouth daily for one month were used. Some animals were fasted prior to injection, while others were fed.

An aqueous solution of alloxan monohydrate containing 20 mg per ml and prepared immediately prior to injection was used. The fasting period was 48 hours in duration. The animals were bled 48 hours after alloxan injection. The blood was obtained by cardiac puncture under nembutal anesthesia. Blood sugar determinations were made on zinc sulfate filtrates according to the Shaffer-Hartman method[10] using reagent No. 50.

In order to determine the effect of alloxan on urine sugar excretion, 4 normal and 2 scorbutic animals weighing from 260 to 490 g were used.

The results obtained are shown in Table I.

In order to determine the effect of alloxan on blood sugar, 26 normal animals varying in weight from 200 to 621 g were used.

The results obtained are shown in Table II.

Three scorbutic animals and 4 scorbutic animals which had received 80 mg chloretone orally for one month were given subcutaneous doses of 1000 mg/kg.

The results obtained are shown in Table III.

The observations of the writers do not confirm the hypoglycemic effects of alloxan in guinea pigs reported by Saviano and De Franciscis. Also we have not observed the mortality after alloxan reported by these workers. Some of the animals have died but many have survived large doses without apparent ill effects. One animal which received 1000 mg/kg subcutaneously is in apparent good health 4 months later. The writers have observed marked variations in the toxicity of alloxan dependent upon the concentration injected. A dosage per kg which administered to rats at a concentration of 20 mg/cc with the production of diabetes and survival of the animal, will often kill the animal if given at a concentration of 40 mg/cc and will have no apparent effect if given at a concentration of 10 mg/cc.

It is of interest to note that the guinea pig is not rendered susceptible to the diabetogenic effects of alloxan by ascorbic acid deprivation, even when this effect is combined with the action of chloretone which presumably increases the demand for ascorbic acid.

Summary. Both the normal and scorbutic guinea pig have been found highly resistant to the production of the diabetic state by alloxan as judged by blood sugar levels and urinary sugar excretion.

[10] Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

16392

Relationship of Hippuric Acid Excretion to Urinary Volume.*

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There is disagreement in the literature in regard to the relationship between hippuric acid excretion and urinary volume. Machella and co-workers[1] have indicated that the amount of hippuric acid excreted in one hour following the injection of sodium benzoate increases as the urine volume rises. Probstein and Londe,[2] on the other hand, using oral administration of sodium benzoate to 14 normal subjects found, by comparing one subject with another, that the amount of hippuric acid excreted was apparently independent of urinary volume. This interrelationship is of considerable clinical importance. In order for the present standards for the interpretation of the hippuric acid test of hepatic function, as it is commonly and widely used, to be valid, the amount of hippuric acid excreted should be independent of urine volume. This report deals with the effects of altering urinary volume in a given subject on the amount of hippuric acid excreted after a standard dose of sodium benzoate.

The intravenous one hour hippuric acid test was performed by the method of Quick,[3] with the modifications suggested by Marron.[4] Of the 9 subjects investigated, 3 were house-staff members, and the others were chosen because of the mildness of the disease for which they were hospitalized and the absence of evidence of liver disease. All of the procedures, including the analyses, were done by one of us (P.S.) in a uniform manner. A series of 3 tests was done on each subject on 3 consecutive days. In each instance, no food was taken for 6 hours prior to the determination. The urine volume was altered by varying the amount of water given to the subject prior to the test. Nausea was never encountered. The subjects were all young men who were allowed to empty their bladders completely while standing erect.

In order to check for impurities in the hippuric acid yields, the crystals in 5 cases were re-dissolved after weighing and titrated with 0.2 N sodium hydroxide, using phenolphthalein as indicator; in no instance was the difference between the results obtained by the gravimetric and the titrametric procedures greater than 0.03 g. As a further precaution, melting point determinations were done on 5 cases. These varied between 182.0°C and 186.0°C. The melting point for pure hippuric acid crystals is given as 187.5°C.

The results are given in Table I. The rate of urine excretion ranged between extremes of 1 and 15 ml per minute without any significant variation in the amount of hippuric acid excreted. In the low urine output group (0 to 2 ml per min.), the average amount of hippuric acid excreted was 1.48 ± 0.67 g. In the high urine output group (5 to 15 ml per min.), the average amount of hippuric acid excreted was 1.54 ± 0.66 g — a difference which falls within the range of experimental error. A medium urine output (2 to 5 ml per min.) was obtained on only 6 of the subjects; the average hippuric acid excretion in this group was 1.42 g.

The lack of correlation between hippuric acid excretion and urine volume suggests that hippuric acid is excreted by the renal tubules, for this independence from volume is one of the identifying characteristics of substances excreted by the tubules. The investigation of

* This work was supported by a grant from the Life Insurance Medical Research Fund.

1 Machella, T. E., Helm, J. D., and Chornock, F. W., *J. Clin. Invest.*, 1942, **21**, 763.

2 Probstein, J. G., and Londe, S., *Ann. Surg.*, 1940, **111**, 230

3 Quick, A. J., Ottenstein, H. N., Weltchek, H., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 77.

4 Marron, T. U., *J. Lab. and Clin. Med.*, 1941, **27**, 108.

TABLE I. Hippuric Acid Excretion at Varying Urinary Volumes.

Subject	Diagnosis	Low urine output (0 to 2 ml/min.)		Medium urine output (2 to 5 ml/min.)		High urine output (5 ml/min. and above)	
		Urine vol. ml/hr	Hippuric acid excr. g/hr	Urine vol. ml/hr	Hippuric acid excr. g/hr	Urine vol. ml/hr	Hippuric acid excr. g/hr
P.S.	Normal	(a) 56	1.71			574	1.53
		(b) 97	1.50				
J.M.	"	72	1.66	129	1.56	658	1.37
A.T.	"	(a) 61	1.90			476	2.06
		(b) 67	2.02				
W.S.	Duodenal ulcer	80	0.95	295	1.29	675	1.09
K.C.	Lung abscess	57	0.79	175	0.98	865	0.86
L.Y.	CNS syphilis, fever therapy	72	1.15	225	1.15	575	1.58
J.E.	Rheumatoid arthritis	116	1.91	260	1.88	875	2.00
T.S.	Bysinosis	75	1.50	155	1.65	900	1.58
W.W.	Hysteria	(a) 60	1.76			600	1.76
		(b) 105	1.85				
	Mean	77	1.48 ± 0.67*	206	1.42 ±0.71*	689	1.54 ± 0.66*

* Standard deviation.

Smith and co-workers,[5] who showed that various hydroxy- and amino-substituted derivatives of hippuric acid are excreted by the renal tubules would support this view. The rapidity of excretion of hippuric acid into the urine[6] is also in favor of its being so excreted by a tubular mechanism.

The independence of hippuric acid excretion and urinary volume would apply, of course, only to subjects with normal or moderately impaired renal function. The normal kidney has considerable reserve in the excretion of hippuric acid in the standard test, as Schwei and Quick[6] have shown that the ability of the kidney to excrete hippuric acid is over 2.5 times as great as the speed of synthesis by the liver. Thus it is not unexpected that normal hippuric acid excretion will occur despite a diminished urea clearance and even in some instances of mild elevation of the blood urea. Theoretically, when renal damage is far advanced, active tubular excretion as well as reabsorption by the tubules of that hippuric acid filtered through the glomeruli would be markedly impaired. Then more hippuric acid could be eliminated in a large than in a small output of urine. That this situation does exist at times is suggested by the report of Snapper and Grunbaum[7] in which patients with uremia did excrete more hippuric acid in larger volumes of urine.

Summary. The rate of excretion of hippuric acid is independent of urine volume, indicating that the present standards for the interpretation of the hippuric acid test are valid. It is suggested that hippuric acid excretion is a function of the renal tubules.

[5] Smith, H. W., Finkelstein, H., Aliminosa, T., Crawford, B., and Grober, M., *J. Clin. Invest.*, 1945, **24**, 388.

[6] Schwei, G. P., and Quick, A. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 319.

[7] Snapper, J., and Grunbaum, A., *Klin. Wchnschr.*, 1924, **3**, 101.

16393

Streptomycin. IV. Adsorption of Streptomycin by Susceptible and Resistant Bacteria.

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It has been shown in another report[1] that streptomycin (SM) interferes with the carbohydrate metabolism of resting bacteria which are susceptible to the antibiotic, whereas the metabolism of resistant cells is essentially unaffected. Consideration was given to the possibility that this interference with oxidative metabolism might be directly related in a causative way to the inhibition of bacterial multiplication. Assuming this interpretation to be correct, it was recognized that there are at least two possible reasons for the resistance of some bacteria to SM: first, resistant cells may have different metabolic systems containing enzymes insensitive to the drug, or, second, the resistant cell membrane may be impermeable to the antibiotic and thus bar its entrance into the cell. The metabolic systems in the latter case would not have to be qualitatively different from those of sensitive cells.

A corollary of the latter postulate is that the antibiotic exerts is action after passing the cell membrane. The alternative is that the antibiotic exerts its activity at the cell surface. In such a case the resistance of an organism to the antibiotic would not involve permeability of the cell membrane so far as the antibiotic is concerned, but might be associated with the adsorptive capacity of the membrane for the antibiotic.

With these ideas in mind an attempt was made to determine whether or not susceptibility to SM is correlated with either permeability of the cell membrane to SM, or the adsorptive capacity of the cell for SM, and whether its failure to inhibit the carbohydrate metabolism of resistant bacteria results from its inability to gain access to the metabolic enzymes.

Adsorption of streptomycin by bacteria. The experiments reported in this section were run with susceptible and resistant strains (derived from the susceptible strains) of *Staphylococcus aureus, Shigella dysenteriae* Sonne, and *Bacillus cereus*. In each instance the resistant strain was able to grow in the presence of 1000 μg SM/ml and the susceptible strain was inhibited by 1 μg/ml. All cells were grown in Difco brain heart infusion broth with PAB at 37°C for 16 hours on an International shaking machine, after which they were centrifuged, washed once with distilled water and then taken up in the desired volume of distilled water. Wherever NaCl was used it was added in the solid form in quantities sufficient to give the desired concentration. The SM used was streptomycin sulfate, Abbott. All SM assays were done by the method of Loo, *et al.*[2]

In one series of experiments bacterial suspensions having a packed cell volume of 20 to 78% were exposed to varying concentrations of SM, without added salt, with 1 M NaCl at the start, and with 1 M NaCl added at intervals of 3/4 to 2 hours after shaking had begun. Packed cell volumes were measured by centrifuging the cell suspensions in Wintrobe tubes at 4000 R.P.M. for 1 1/2 hours. All exposures of the cells were carried out at 37°C. After 2 to 4 hours exposure to SM the suspensions were centrifuged and the supernatants decanted and assayed for SM. The cells were then resuspended in the same

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1 Henry, J., Henry, R. J., Housewright, R. D., and Berkman, S., to be published.

2 Loo, Y. H., Skell, P. S., Thornberry, H. H., Ehrlich, J., McGuire, J. M., Savage, G. M., and Sylvester, J. C., *J. Bact.*, 1945, **50**, 701.

TABLE I.

The Combination of Streptomycin with Susceptible and Resistant Strains of *Staph. aureus:* Its Prevention by 1 M NaCl and Poor Elution with Water.

	Packed cell vol. %	Streptomycin μg/ml fluid at 0 time	Streptomycin recovered after shaking at 37°C: 3 hrs μg/ml	3 hrs %	Overnight elution of cells with water μg/ml	Overnight elution %	Total streptomycin recovered, %
Susceptible strain (S) + streptomycin	25	67	12	18	12	18	36
S + 1 M NaCl ¾ hr after shaking started	25	67	55	82	12	18	100
S + 1 M NaCl at start of experiment	25	67	58	87	8	8	95
Resistant strain (R) + streptomycin	23	65	7	11	13	20	31
R + 1 M NaCl ¾ hr after shaking started	23	65	53	82	12	19	101
R + 1 M NaCl at start of experiment	23	65	53	82	8	12	94

TABLE II.

Combination of Streptomycin with Susceptible and Resistant Strains of *Staph. aureus:* Its Prevention and Elution with 1 M NaCl.

	Packed cell vol. %	Streptomycin μg/ml fluid at 0 time	Streptomycin recovered after shaking at 37°C: 4 hrs μg/ml	4 hrs %	Overnight elution of cells with 1 M NaCl μg/ml	Overnight elution %	Total streptomycin recovered, %
Susceptible strain (S) + streptomycin	41	85	35	41	41	48	89
S + 1 M NaCl 2 hrs after shaking started	41	85	75	88	18	21	109
S + 1 M NaCl at start of experiment	41	85	68	80	21	25	105
Resistant strain (R) + streptomycin	38	80	25	31	45	56	87
R + 1 M NaCl 2 hrs after shaking started	38	80	77	96	20	25	121
R + 1 M NaCl at start of experiment	38	80	67	84	20	25	109

TABLE III.
Correlation of the % Cell Suspension of Susceptible and Resistant Strains of *Staph. aureus* with Removal of Streptomycin from Solution.

	Packed cell vol., %	Streptomycin μg/ml fluid at 0 time	Streptomycin recovered after 3 hrs	
			μg/ml	%
Susceptible strain	60	115	21	18
" "	6	49	10	20
" "	0.6	46	32	70
" "	0.06	46	45	99
" "	0.006	46	42	91
Resistant strain	55	98	14	14
" "	5.5	51	9	18
" "	0.5	46	32	70
" "	0.05	46	45	99
" "	0.005	46	40	87

volume of distilled water or 1 M NaCl and placed overnight at 37°C on an International shaking machine. Again, following centrifugation, the supernatants were assayed for SM. Equilibrium between the SM and the bacterial cells in the initial suspension apparently was at least closely approached within 3/4 hour, since the amount of SM removed from solution was constant within experimental error when the time of exposure was varied from 3/4 to 4 hours. Results of typical experiments run under these conditions are shown in Tables I and II. The former table shows that recovery of SM from *Staph. aureus* cells was low in the absence of NaCl and very high in its presence whether the salt was added with the SM or 3/4 hour after the experiment was begun. When water was used to elute these cells overnight practically all the remaining SM was recovered in those instances where the cells had been exposed to salt, while about 65% of the SM was not recovered from those cells not exposed to salt.

The experiment in Table II was run in the same fashion as that in Table I except that in 1 case 1 M NaCl was added to the cells 2 hours after exposure to SM and the cells were eluted overnight with 1 M NaCl instead of with distilled water. Here, using NaCl in the eluting fluid, a great deal more of the SM was recovered from cells not originally exposed to NaCl than when distilled water was used as the eluant. Within experimental error of the test methods used recoveries of SM in the former case always totaled 100%. The totals of some of the recoveries aggregated more than 100%. Presumably this was caused by supernatant fluid not removed by decantation and by fluid remaining in the interstices of the packed cells.

In general it was found that SM was removed from solution to the same extent by both susceptible and resistant strains of the same species of bacteria. The experiments described indicate that considerable amounts of SM are taken up from water solution by heavy suspensions of both resistant and susceptible bacteria.

It seemed of interest to determine the % cell suspension at which the decrease in SM concentration is too small to be detected by the assay method used. These experiments were run by making 5 10-fold dilutions of a heavy suspension of organisms. All the suspensions were exposed to water solutions of SM for 2 to 3 hours at 37°C on an International shaking machine. Table III shows the results of 1 such experiment using susceptible and resistant strains of *Staph. aureus*. It can be seen that as the volume of cells decreased the recovery of SM increased. When the packed cell volume was small (0.05% in this case) recovery was complete within the experimental error of the assay method. The assay method is not sufficiently precise to detect the small decreases in SM concentration resulting from contact with such a relatively small number of cells. With a 60% suspension of cells, on the other hand, there was only 18% recovery of SM. Essen-

tially the same results were obtained with susceptible and resistant strains of the other 2 organisms.

In the presence of bacteria, therefore, SM decreases in concentration in the supernatant. If the cellular membrane is permeable to SM, the latter should distribute itself between the cellular contents and the surrounding medium according to its relative solubility in each of the 2 phases. At a packed cell volume of 0.5%, 30% of the SM disappeared from solution (Table III). If the only cause for this decrease was a greater solubility in the cellular protoplasm, the distribution coefficient would be far from unity, a situation which is very improbable. When 2% NaCl was added with the SM, the cells took up a negligible amount of SM. This indicates that unless salt *per se* blocks entrance of SM into the cell, little or no SM gets in. Such a salt block seems unlikely, since it is difficult to conceive how a distribution coefficient could be so markedly changed by the presence of salt. If the cells were first allowed to take up the SM and then salt added, followed by one elution with water or saline, there was total recovery of the SM in the combined first supernatant and eluate. This is interpreted to mean that little or no SM penetrated the cell membrane.‡ Otherwise one must assume that in the presence of salt the cell excreted SM from the cell against a concentration gradient, which would seem extremely questionable.

All or nearly all of the SM taken up by bacteria would appear, therefore, to be in the adsorbed state at the cell surface. The elution of this SM by salt confirms this hypothesis. SM adsorption and its elution by salt has been observed in inanimate systems,[4] and salts in general antagonize the bacteriostatic action of SM.[5]

Studies on the rate of desorption of SM from bacterial cells and its possible relation to the "persistent" action of SM. Recently reports have begun to appear in the literature indicating a fallacy in the assumption that a certain minimal blood concentration of an antibiotic must be maintained for effective therapy. At least one such report has appeared concerning SM in the treatment of tuberculosis in guinea pigs.[6] Apparently the inhibitory action of the antibiotic can persist after the blood concentration falls below the sensitivity of the test for the antibiotic. If the rate of desorption of SM from bacteria in water is very slow, it was believed that this reported phenomenon of persistent action might be explained as follows: when the blood concentration is high the SM is adsorbed by the bacteria; when the blood level falls to zero the SM adsorbed by the bacteria is still there because of a slow rate of desorption, and it is presumably situated so as to effect an inhibition. The concentration of salts present in body fluids and tissues would not be sufficiently great to prevent completely such an adsorption.

As seen in Table I (and in all other similar experiments) the ratio of SM recovered in the first supernatant to that adsorbed is of the same order of magnitude as the SM recovered in the water eluate to that remaining adsorbed. As previously stated some of the SM in the water eluate undoubtedly originates from the first supernatant. Since this amount cannot be calculated with accuracy, it cannot be determined whether or not these ratios are the same. From these data, therefore, it was impossible to determine the rate of desorption of SM from the bacterial cells in the presence of water. Experiments were designed to investigate further the possibility that the persistent action may be due to a slow rate of desorption of SM from bacterial cells.

‡ It has been reported[3] recently that SM forms a colloidal sol and not a true solution in distilled water. The particle size of the dispersed phase was estimated to be approximately 65 mμ. The fact that SM passes freely through cellophane membrane[4] indicates that these large aggregates must be in equilibrium with particles of much smaller molecular size.

[3] Hauser, E. A., Phillips, R. G., and Phillips, J. W., *Science*, 1947, **106**, 616.

[4] Berkman, S., Housewright, R. D., Henry, R. J., and Henry, J., to be published.

[5] Berkman, S., Henry, R. J., and Housewright, R. D., *J. Bact.*, 1947, **53**, 567.

[6] Corper, H. J., and Cohn, M. L., *Science*, 1947, **106**, 446.

In one series of experiments, susceptible cells of *B. cereus* B-569 (packed cell volumes of 25 to 30%) were exposed to SM (400 to 1100 μg/ml) in water and in 1 M NaCl for 2 hours at 37°C on an International shaking machine. Aliquots were then placed in cellophane dialysis bags and dialyzed against running water and 1 M NaCl respectively. At intervals during a 48 hour period a set of dialysis bags was removed, the suspensions centrifuged, the supernatants assayed for SM, and the cells resuspended in 1 M NaCl. After 16 hours this salt eluate was also assayed for SM.

In the case of exposure of cells to SM in water followed by dialysis versus water, SM could be recovered in decreasing amounts over the period of 48 hours both in the supernatant and in the NaCl eluate. Approximately 4 times as much SM was recovered in the eluate as in the supernatant at each time interval. In the case of exposure of cells to SM in 1 M NaCl followed by dialysis versus 1 M NaCl solution, either no SM or very minute amounts of SM could be recovered at 24 hours from the supernatant. The fact that in the latter case practically all the SM was lost by dialysis within 24 hours indicates that the SM concentration in the supernatants of cells dialyzed versus water was being maintained by continuous desorption of the SM from the cells in the presence of water. These results confirm the earlier observations on the adsorption of SM by bacteria and show that SM does desorb in the presence of water.

In the above experiments the criterion of adsorption and elution of SM from bacteria was indirectly determined by estimation of the antibiotic in the supernatants. In another series of experiments, adsorption of SM was determined by whether or not the cells were able to multiply, *i.e.*, it was assumed that as long as SM was adsorbed at the critical site or sites of action of SM, the cells would be unable to divide. Cells of the susceptible strain of *B. cereus* B-569 were exposed to concentrations of SM ranging from 10 to 1000 μg/ml for periods of time up to 48 hours. The cells were then counted by the pour plate method in nutrient agar and in nutrient agar containing 1.5% NaCl. The dilutions required to make the plate counts reduced the SM concentrations far below the minimal inhibitory level. Although there was a decided drop in bacterial count after 2 hours exposure to SM, presumably due to its bactericidal action, counts made at intervals over the periods of the experiments were essentially the same in the presence and absence of NaCl. Since in controls this concentration of NaCl completely antagonized the action of 10 μg SM/ml it seems necessary to conclude that, in this case at least, that part of the adsorbed SM responsible for its inhibitory action easily desorbs on exposure to water.

It is possible that SM is adsorbed by one or more constituents of the agar in which the cells with their adsorbed SM were plated out for counting. Such an adsorption by the agar should not affect the affinity between SM and the bacterial cells but might affect the rate of desorption from the cells by quickly binding the SM as it desorbs from the cells and thus keeping the concentration of free SM at a very low level. It does not seem probable that this could be a decisive factor in the desorption of SM from the cells in the agar since the concentration of SM surrounding the cells at the time of plating has been reduced tremendously in the dilution procedure required for making counts and since the actual amount of SM adsorbed per cell is extremely minute. If such an adsorption between agar and SM does not occur, free SM as it desorbs from the bacteria would diffuse into the surrounding medium. Since visible colonies appeared on the plates after approximately the same duration of incubation whether the cells had or had not been exposed to SM, apparently desorption proceeded at a sufficiently rapid rate to permit the cells to begin multiplying almost as soon as untreated cells.

These observations, therefore, are not compatible with the hypothesis that the persistent action of SM *in vivo* is a result of slow desorption of SM from the adsorbed state on bacterial cells.

Summary and Conclusions. Studies with *Staph. aureus, S. dysenteriae* Sonne, and *B.*

cereus indicate that streptomycin is adsorbed by bacterial cells, all or nearly all of the adsorption occurring at the cell surface. There was no demonstrable difference in adsorption between susceptible and resistant strains of these organisms. The affinity of streptomycin for bacterial cells is greatly reduced in the presence of NaCl. Evidence is presented indicating that the persistent action of streptomycin *in vivo* after the blood level falls below a detectable value is not related to a slow desorption of streptomycin from the adsorbed state on bacterial cells.

16394 P

An Outbreak of Equine Encephalomyelitis, Eastern Type, in Southwestern Louisiana.

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From May to October of 1947 an outbreak of encephalomyelitis occurred among horses and mules in southwestern Louisiana. Thousands were implicated with approximately 3,713 deaths.[1] The disease began in Iberia Parish, spread west to Texas and northwest to DeSoto Parish. Because the eastern type of equine encephalomyelitis virus was recovered from human cases that occurred coincidentally, a preliminary report is submitted.

The eastern equine virus was first isolated from horses in 1933,[2,3] and from children in Massachusetts during 1938.[4,5] Antibodies were found in the serum of an adult in 1939.[6] All cases of the eastern type were thought to be confined to the Atlantic coastline until 1940[7] and 1941[8] when it was recovered from horses in both Alabama and Texas. Subsequently equine cases have been reported in all of the Gulf States;[9] also in Missouri,[9] Michigan,[10] Mexico,[11] Brazil[12] and Panama.[13] Besides the Massachusetts outbreak the only human cases ascribed to the eastern strain were 3 in Texas[14,15] that showed neutralizing antibodies in the serum and 1 in Louisiana from whom the virus was recovered in 1946.[16]

In the present outbreak all human cases except one were in children from 7 months to 15 years of age. The one fatal adult case

1 Report by the Bureau of Animal Industry, *Vet. Med.*, 1947, **42**, 399.

2 TenBroeck, C., and Merrill, M. H., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 217.

3 Giltner, L. T., and Shahan, M. S., *North Am. Vet.*, 1933, **14**, 25.

4 Fothergill, LeR. D., Dingle, J. H., Farber, S., and Connerly, M. L., *New England J. Med.*, 1938, **219**, 411.

5 Feemster, R. F., *Am. J. Pub. Health*, 1938, **28**, 1403.

6 McAdams, J. C., and Porter, J. E., *New Eng. J. Med.*, 1939, **221**, 163.

7 Mohler, J. R., Report of the Chief of the Bureau of Animal Industry, U. S. Dept. Agriculture, 1940.

8 Randall, R., and Eichorn, E. A., *J. Am. Vet. Med. Assn.*, 1941, **98**, 448.

9 Shahan, M. S., and Giltner, L. T., *J. Am. Vet. Med. Assn.*, 1945, **107**, 279.

10 Brown, G. C., *J. Inf. Dis.*, 1947, **81**, 48.

11 Tellez, G. A., *Rev. Inst. Pecuario*, 1941, **1**, 36.

12 Lennette, E. H., and Fox, J. P., *Memorias Inst. Oswaldo Cruz*, 1943, **38**, 1.

13 Steele, J. H., and Habel, K., *J. Am. Vet. Med. Assn.*, 1947, **111**, 263.

14 Bohls, S. W., and Irons, J. V., *Texas State J. Med.*, 1942, **38**, 260.

15 Hammon, W. McD., *J.A.M.A.*, 1943, **121**, 560

16 Kelly, G. P., Milner, K. C., and Shaffer, M. F., *New Orleans Med. and Surg. J.*, 1947, **100**, 270.

TABLE I.
Results of Serum Neutralization Tests.

Source of sera	E.E.E. virus			W.E.E. virus		
	No. tested	No. positive	% positive	No. tested	No. positive	% positive
Human						
Died	6	1 pos. 2 wkly*	50	6	0	0
Recovered	5	3	60	5	2 wkly	40
Contacts	22	4	18.1	16	0	0
Neighbors	23	0	0			
Horses and Mules						
Recovered	11	8	72.7	11	2 pos. 1 wkly	27.2
Sick	4	4	100	4	1 pos. 3 wkly	100
Normal	14	9	64.2	12	1 "	8.3
Cows	5	2 wkly	40.0	3	2 "	66.6
Chickens	105	2 pos. 2 wkly	3.8	88	19	21.5
Ducks	8	0	0	8	0	0
Geese	8	1 wkly	12.5	8	0	0
Turkeys	3	0	0	3	0	0
Pigeons	35	1 wkly	2.8	30	0	0
Pig	1	0	0	1	0	0
Dogs	3	1	33.3	3	1	33.3

* wkly = weakly positive.

was aged 74. The patients were from rural or small town areas; many mosquitoes were observed and most of the houses were unscreened.

Case history of E. R. D. The following typical case history is submitted. A 2-year-old white girl from Maurice, Louisiana, became restless, irritable, drowsy, febrile and had several generalized convulsions. The following day a lumbar puncture revealed a lymphocytic pleocytosis. She was taken to New Orleans Charity Hospital, where examination revealed an acutely ill, febrile, comatose child, having frequent convulsions. There was stiffness of neck, back, and upper and lower extremities. Despite chemo-and supportive therapy the patient became progressively worse with almost continuous convulsions, until death occurred on the fifth day of illness. The virus of eastern equine encephalomyelitis was recovered from the brain.

Laboratory findings. Fresh brain material was obtained from 6 fatal equine cases, 3 of them through the courtesy of the State Veterinary Department and from 8 human fatalities through the courtesy of the Louisiana State Health Department and the Tulane Service of Charity Hospital, New Orleans.

Brain suspensions were inoculated intracerebrally and intraabdominally into guinea pigs and 10-day-old white mice with recovery of a virus from 2 human and 3 equine tissues. It could be passed through Mandler and Seitz filters, was lethal for mice, guinea pigs and hamsters in 48 hours and for embryonated eggs in 24 hours. The mice showed a tremor, drowsiness, hyperexcitability followed by convulsions. The guinea pigs exhibited a temperature of 106°C and a rhythmic trotting motion of all 4 extremities. Identification was accomplished by means of the neutralization test in mice. All new strains were neutralized by immune serum of the eastern equine virus and not by that of the western and the St. Louis encephalitis. Hyperimmunized guinea pigs were inoculated intracerebrally. Those animals immune to the eastern equine virus remained alive while the normal controls and guinea pigs immune to the western strain died in 48 hours. Serum from a recovered guinea pig given human brain material contained complement fixing antibodies against the eastern but not the western equine antigen.

Neutralization tests against the eastern and

western equine viruses were performed with the sera of equine and human recovered cases and their contacts and also with sera from domestic animals found on the farms of fatal cases. The results are given in the Table I. Besides the positive results with the human and horse sera only 2 chickens and 1 dog showed strong antibodies for the eastern virus. The sera of 2 chickens, 1 goose, 1 pigeon and 2 cows were only weakly positive. Many of these tests were performed with one of the newly isolated human strains of virus. It was of interest that 25.9% of the unvaccinated horses, 18.1% of the chickens and 1 dog had antibodies for the western equine virus, while the sera of 2 cows and 2 people were weakly positive.

The histopathology in the horse brains showed focal and diffuse gliosis, perivascular round-cell infiltration and edema. The motor neurons showed chromatolysis with cytolysis and phagocytosis by the glial cells. Passage of a human strain into guinea pigs produced severe neuron degeneration and mild perivascular infiltration with edema.

Arthropods were collected from the different areas where encephalitic cases occurred. A later report will be presented as well as a more detailed epidemiological account of this outbreak. The clinical cases will be described by Dr. J. Syverton of Louisiana State University.

Summary. The virus of eastern equine encephalomyelitis was recovered from the brains of both human and equine fatal cases occurring in an outbreak of encephalomyelitis in southwestern Louisiana. Positive neutralization tests to this virus were obtained with sera of 2 chickens, 1 dog, and 31 human and equine cases and their contacts, while those of 2 chickens, 1 goose, 1 pigeon and 2 cows were only weakly positive. Antibodies to the western equine virus were found in the sera of 1 dog, 8 horses and 19 chickens and to a very slight degree in the sera of 2 humans and 2 cows.

16395

Cold Hemagglutinin in Chinese Kala Azar.

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Rose[1] observed high titers of cold hemagglutinin in two cases of kala azar and concluded that such agglutinin developed as a result of the parasitic infection. This observation has been studied in order to determine its possible clinical significance in this disease.

Method and Material. Collection of blood specimens and the titration of the cold hemagglutinin content in fresh serums were carried out strictly according to Rose's method.[1] In nearly all of the tests reported in this paper, group O erythrocytes from one of us, Hou, were used. The final dilution of the serum in the last tube showing fine granular clumpings was recorded as the titer of each test. The serums studied included specimens from 68 proven kala azar patients, 62 normal individuals, and 12 atypical pneumonia cases.

Results. The results of the above mentioned investigations are summarized in Table I.

a. *Normal controls.* Cold hemagglutinin with titers varying from 1:4 to 1:32 was present in 56.4% of 62 normal individuals, but absent in the remaining 43.6%. The highest titer recorded in our normal controls was 1:32. Therefore in the present study only titers above 1:32 have been considered significant.

b. *Primary atypical pneumonia cases.* This

[1] Rose, H. M., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 93.

TABLE I.
Distribution of Cold Hemagglutinin Titers in 142 Subjects.

Clinical group	No. of subjects	No. of tests	Cold hemagglutinin titers								
			Neg.	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Normal control	62	66	30	9	14	9	4	0	0	0	0	0
Kala azar	68	140	54	18	26	17	13	6	5	1	0	0
Atypical pneu.	12	20	0	0	1	1	0	5	5	6	1	1

series of cases served as positive controls. Cold hemagglutinin with titers above 1:32 was present in all of the 12 cases (100%) so diagnosed. The highest value obtained was 1:1024.

c. *Kala azar cases.* Among 68 patients with kala azar, only 11 cases (16.2%) gave cold hemagglutinin titers above 1:32, while 41 patients (60.3%) showed lower titers indistinguishable from those of normal individuals. The highest titer was 1:256.

Discussion. Although cold hemagglutinin can be demonstrated in almost every infectious disease, high titers have been rarely encountered except in a few diseases such as primary atypical pneumonia, infectious mononucleosis, trypanosomiasis, measles, mumps orchitis, and scarlet fever.[2,3,4] It might be expected that kala azar, a protozoal infection, like trypanosomiasis, might give high cold hemagglutinin titers as claimed by Rose.[1] The results of our study, however, failed to confirm this. Furthermore, the results of repeated observations made in the course of illness of 68 kala azar patients, indicate that the cold hemagglutinin titer bears no relationship to the severity, stage, and treatment of the disease. For instance, 12 cases complicated by noma (*Cancrum oris*) showed no particular increase in the hemagglutinin titer. Similarly, another fatal case also failed to give a high titer. Of some interest is the fact that one patient, after complete cure for more than one year, still showed a titer 1:64.

As there is some evidence that cold hemagglutinin is an immune body, closely related to serum globulin,[4] our data in 33 kala azar cases were analyzed to see if there is any correlation between cold hemagglutinin titer and serum globulin content. Results shown in Table II failed to demonstrate any such correlation.

Summary. Cold hemagglutinin in titers above 1:32 has been found in only 16.2%

2 Stats, D., and Wassermann, L. R., *Medicine*, 1943, **22**, 363.

3 Young, L. E., *Am. J. Med. Sci.*, 1946, **211**, 23.

4 Spingarn, C. L., and Jones, J. P., *Arch. Int. Med.*, 1945, **76**, 75.

TABLE II.
Correlation Between Cold Hemagglutinin Titer and Serum Globulin Content of 33 Kala Azar Cases.

Serum globulin content, g%	Cold hemagglutinin titers							
	Neg.	1:4	1:8	1:16	1:32	1:64	1:128	1:256
2.1-3				1	1			
3.1-4	5	2	1	1	2			1
4.1-5	1	2	5					
5.1-6	3		3				1	
6.1-7	1	1						
7.1-8			1				1	

among 68 Chinese patients with kala azar. The highest titer obtained was 1:256. The titer bears no relationship to the severity, stage, and treatment of the disease. Our results further suggest that there is no correlation between cold hemagglutinin titer and serum globulin content.

16396

Influence of Repeated Anoxia, Electroshock and Insulin Hypoglycemia on Reactivity of Sympathetico-Adrenal System.*

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Numerous investigations[1] have been published by the senior author indicating that procedures such as electrically or chemically induced convulsions, insulin coma and anoxia which are used in the "shock therapy" of mental disease involve an excitation of the sympathetico-adrenal and vago-insulin systems in which the effect on the former system predominates.[2] Further experiments[3] showed that stimulation of the sympathetic division of the hypothalamus has a profound influence on cortical activity. This suggested the possibility that procedures leading to sympathetic discharge and increased reactivity of sympathetic autonomic centers may alter cortical activity not only during the experimental procedures but also for some time afterwards. Consequently, experiments were performed in which the behavioral changes following repeated insulin coma and similar procedures were studied by means of the conditioned reflex method. These studies showed that repeated insulin comas or repeated convulsions led to the restitution of previously inhibited conditioned reactions.[4] This result was interpreted to mean that increased discharges originating in the hypothalamus persisted for some time after the animals had been subjected to coma and convulsions and that these discharges altered the reactivity of those cortical centers which were involved in conditioned reflexes.

If this interpretation is correct, it should be possible to relate this effect to the persistence of increased autonomic reactivity.

* Aided by a grant from the Office of Naval Research.

1 Gellhorn, E., *Arch. Neurol. and Psychiat.*, 1938, **40**, 125.

2 For a summary of our earlier work *cf.* Gellhorn, E., *Autonomic Regulations*, New York, 1943.

3 Murphy, J. P., and Gellhorn, E., *J. Neurophysiol.*, 1945, **8**, 341, 431.

4 Gellhorn, E., and Minatoya, H. J., *J. Neurophysiol.*, 1943, **6**, 161; Kessler, M., and Gellhorn, E., *Am. J. Psychiat.*, 1943, **99**, 687; Gellhorn, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 155; *Arch. Neurol. and Psychiat.*, 1946, **56**, 216; PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 375; *Arch. Neurol. and Psychiat.*, 1948, **40**, 125.

TABLE I.
Effect of Anoxia* on Blood Sugar (mg %) of Rats Before and After Repeated Exposure to Low Barometric Pressure.†

No.	Conditions	0′	25′	50′	75′	100′	125′
9	Before repeated exposure	84	93	84	75	73	72
9	After 10 exposures to 350 and 295 mm Hg	80	89	94	86	81	84
9	After 9 additional exposures to 295 mm Hg	75	86	91	102	107	104
9	After a rest of 2 weeks	80	89	84	80	81	84

* Five 25-min. periods at a pressure of 280 mm Hg.
† Exposure to 350 and 295 mm Hg for 4 hours on 5 days of the week.

TABLE II.
Effect of Anoxia* on Blood Sugar (mg %) of Rats Before and After Repeated Electroshocks.

No.	Conditions	0′	25′	50′	75′	100′	125′
10	Before electroshock	84	89	87	79	80	74
10	After 10 daily electroshocks	100	112	106	103	102	102
10	After 14 additional electroshocks in 30 days	81	103	106	104	100	98
10	After a 30-day rest period	88	97	90	88	87	90

* Five 25-min. periods at a pressure of 280 mm Hg.

The present work was undertaken in order to determine, at least indirectly, the state of the sympathetico-adrenal system in animals repeatedly subjected to anoxia, electro-shock and insulin hypoglycemia.

Method. The reactivity of the sympathetico-adrenal system to anoxia was determined before and after subjecting the rats (Sprague-Dawley) to 3 different procedures resembling the shock therapy of mental disease. This anoxia test consisted of an initial determination of blood sugar, and subsequent determinations at 25′ intervals after the rats had been exposed to a barometric pressure of 280 mm Hg for 5 consecutive periods of 25′ each. For further details see the tables and our earlier studies.[5]

Results. I. *The effect of repeated exposures to low barometric pressure on the reactivity of the sympathetico-adrenal system.* Table I summarizes the experiments on the influence of repeated anoxia and shows the following effects:

1. The fasting blood sugar decreases slightly as a result of repeated exposures to anoxia confirming the work of Sundstroem and Michaels.[6]

2. The rise in blood sugar induced by the anoxia test increases as a result of repeated prolonged exposures to low barometric pressure.

3. This effect disappears after a rest period of 2 weeks.

It was pointed out earlier[7] that the effect of anoxia on the blood sugar is a result of the action of low oxygen tension on the centers of the sympathetico-adrenal and vago-insulin systems. In order to avoid misleading conclusions based on averages involving a relatively small number of animals, and to show alterations in the balance of the 2 systems during the course of the experiments the records of each individual animal is presented in Fig. 1 in a form which permits one to see relative predominance in the reactivity of the sympathetico-adrenal system. For this purpose the individual blood sugar curves were graphed and the weight of the graph paper corresponding to the hypoglycemic area was deducted from that representing the hyperglycemic area, the null point being the initial blood sugar. The difference (in mg) is indicated on the ordinate while the abscissa indicates the various stages of the experiment

[5] Safford, H., and Gellhorn, E., PROC. SOC. EXP. BIOL. AND MED., 1947, **60**, 98, 247.

[6] Sundstroem, E. S., and Michaels, G., The Adrenal Cortex in Adaptation to Altitude, Climate. and Cancer, *Memoirs University of California,* 1942, **12**.

[7] Feldman, J., Cortell, R., and Gellhorn, E., *Am. J. Physiol.*, 1940, **131**, 281.

TABLE III.
Effect of Anoxia* on Blood Sugar (mg %) of Rats Before and After Repeated Insulin Comas.

A—Control							B—After exposure to 11 insulin comas in 28 days						
Rat No.	0′	25′	50′	75′	100′	125′	Rat No.	0′	25′	50′	75′	100′	125′
336	90	102	110	99	96	92	336	110	120	108	106	102	102
337	94	110	115	106	99	90	337	99	116	106	94	87	88
339	92	99	112	110	96	87	339	99	96	82	84	82	82
340	94	110	112	96	94	82	340	100	111	94	94	82	84
341	94	122	106	102	99	96	341	110	118	115	111	102	96
343	84	92	94	82	80	80	343	99	94	115	94	90	94
344	94	134	118	110	106	99	344	106	110	106	97	96	92
345	92	112	90	90	82	82	345	102	87	87	83	72	72
Avg	91	110	107	99	94	87	Avg	103	107	101	95	89	89

* Five 25-minute periods at a pressure of 280 mm Hg.

TABLE IV.
Effect of Adrenalin on Blood Sugar (mg %) of Rats in Different Experimental Conditions.

No.	Condition	0′	30′	60′	90′	Adrenalin
8	Control	92 (100)†	119 (130)	111 (122)	97 (106)	1 cc/kg
8	After 10 electroshocks in 10 days	95 (100)	121 (127)	110 (116)	100 (105)	1:50,000 i.p.
8	Control	83 (100)	137 (165)	122 (146)	107 (129)	1.0 cc/kg
8	After 12 insulin* comas in 15 days	107 (100)	117 (109)	114 (106)	116 (108)	1:25,000 i.p.
8	After a rest of 30 days	90 (100)	132 (147)	124 (138)	112 (124)	
10	22 hr fast	89 (100)	119 (133)	108 (122)	96 (107)	1 cc/kg
10	4 ” ”	115 (100)	153 (133)	138 (120)	119 (104)	1:50,000 i.p.

* 0.3 unit insulin/kg i.p.
† The number in ( ) is the percentage of the control.

(control test before and after exposure of the rat to a varying number of prolonged periods of anoxia). If the values are below the zero line the reactivity to the anoxia test reveals a predominating vago-insulin response whereas numbers above the base line indicate predominance of the sympathetico-adrenal system. Fig. 1 shows that repeated periods of exposure to low barometric pressure greatly alters the autonomic balance in favor of the sympathetico-adrenal centers. This holds for all animals no matter whether they show under control conditions a predominance of the vago-insulin or of the sympathetico-adrenal system. These effects were reversible in all but one rat.

In a second series of experiments the rats were subjected to repeated electroshocks causing tonic-clonic convulsions by applying a 60 cycle current of 30 ma for 2 seconds. Table II shows that after 10 electroshocks the fasting blood sugar is markedly elevated, but the hyperglycemic effect of the anoxia test is not greatly altered. However, after 14 more electroshocks the resting blood sugar returned to normal values and the average hyperglycemic response in the anoxia test was markedly increased. Moreover, the blood sugar remained above the control value even in the 125 minute sample, whereas in the control series† the slightly hyperglycemic response had reverted into a hypoglycemic reaction. Finally, a control test taken 30 days after cessation of the electroshock treatment showed that the hyperglycemic response had returned approximately to the control level. Fig. 2 shows the individual records which permit their evaluation in terms of sympathetic or parasympathetic balance. The curves indicate that in 8 out of 10 rats the relative responsiveness of the sympathetico-adrenal system increases slightly after 10 convulsions. After 14 more convulsions all animals show a marked sympathetico-adrenal

†*I.e.*, before application of repeated electro shocks.

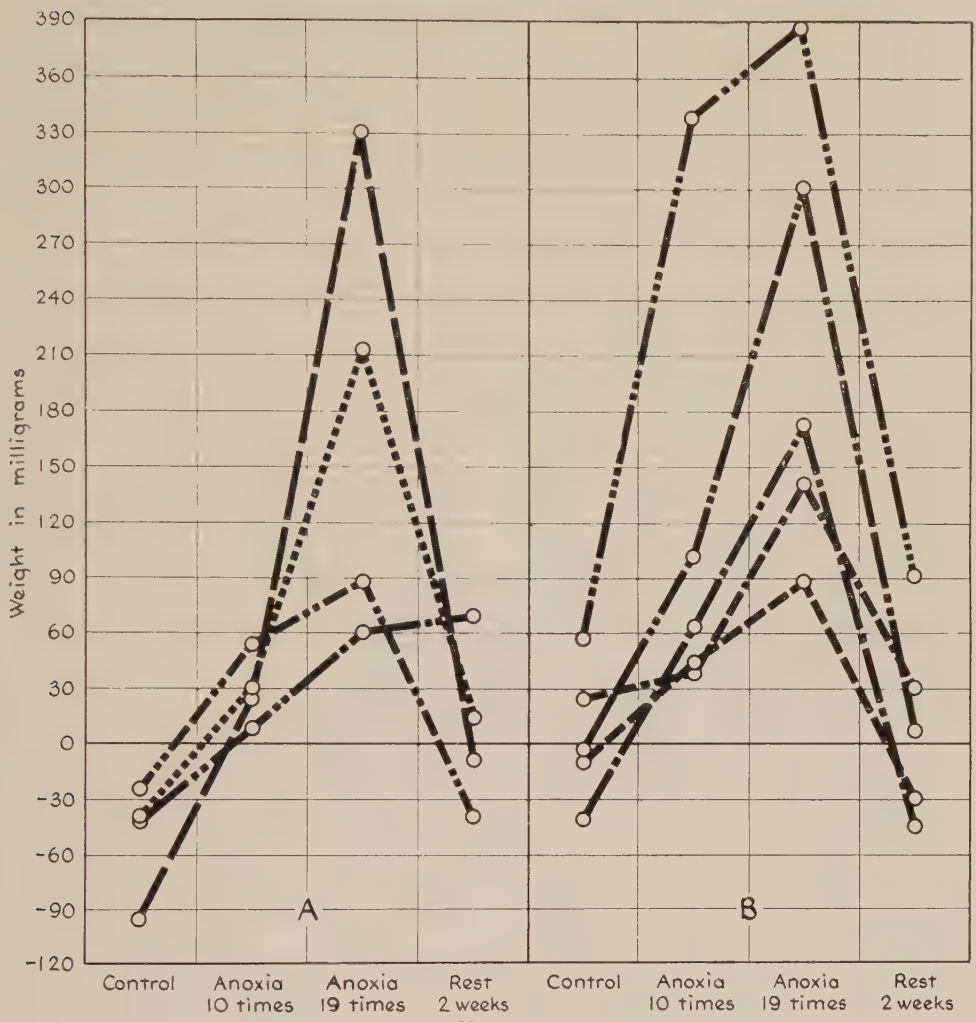

FIG. 1.

The effect of repeated anoxia on the autonomic balance. The blood sugar curve to a standard anoxia test was graphed for each animal and the weight of the area of hypoglycemia was deducted from that of the area of hyperglycemia. Positive values indicate sympathetico-adrenal, negative figures vago-insulin predominance. The figures are divided into A and B in order to avoid superimposition of the curves.

predominance. An interval of 30 days sufficed to restore the original balance of the autonomic centers. In one animal which had shown a marked vago-insulin predominance in the control test taken prior to the administration of the series of convulsions the rest period of 30 days had not only abolished the sympathetico-adrenal predominance caused by the convulsions but actually aggravated the vago-insulin predominance found under control conditions.

The third series of experiments concerns the effect of repeated insulin comas on the autonomic balance as tested by the blood sugar curve resulting from the anoxia test. Table III shows the results on 8 rats before and after exposure to 11 insulin comas. The initial blood sugar values rise after repeated comas, but the hyperglycemic reaction in response to the anoxia test is abolished. The individual blood sugar curves of Table III and particularly their evaluation in terms of autonomic balance by the methods previously employed in Fig. 1 and 2 showed that in all

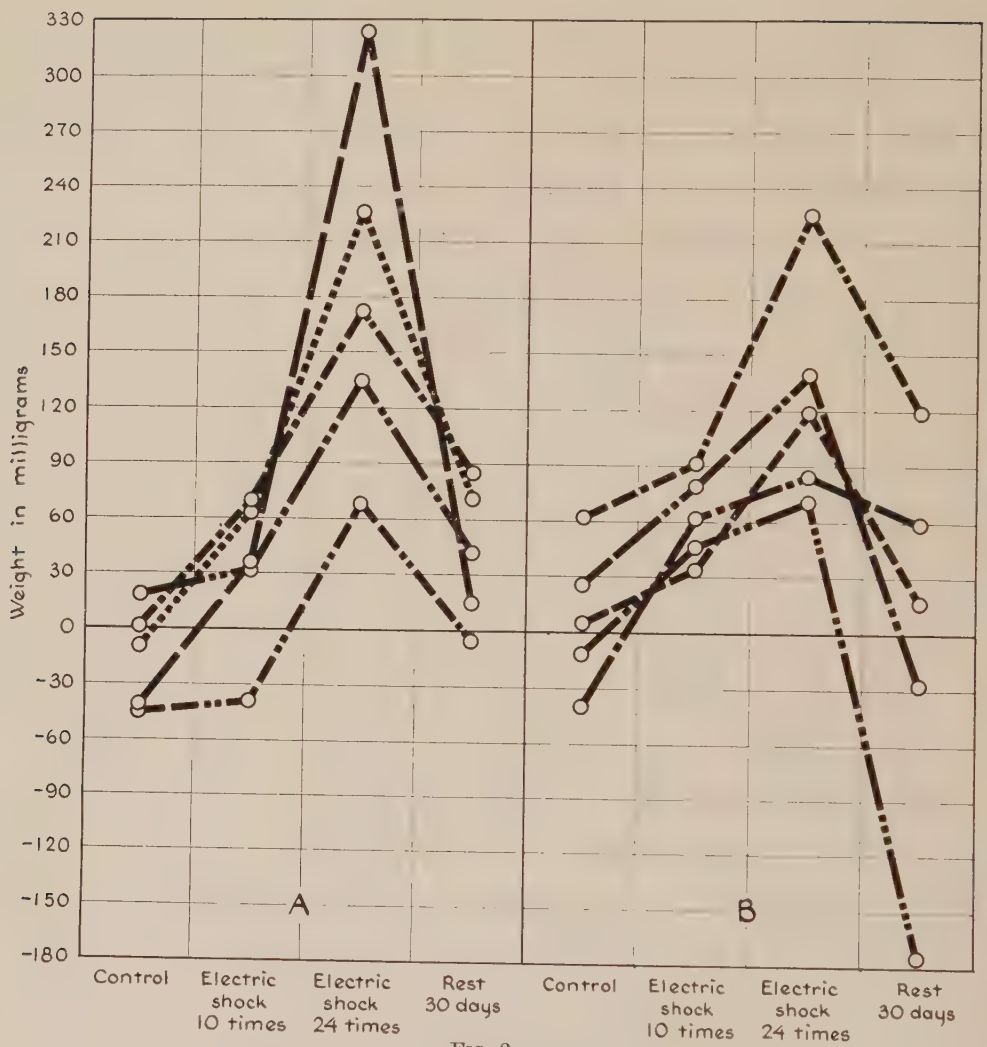

FIG. 2.
The influence of repeated electroshocks on autonomic balance.*

instances the sympathetico-adrenal predominance existing under control conditions was abolished and that in about 50% of the cases the vago-insulin system became dominant.

This striking and unexpected finding could have several causes. One would think primarily of an increased reactivity of the centers of the vago-insulin system, a decreased reactivity of the sympathetico-adrenal system or of a failure of the liver to react to adrenalin with increased glycogenolysis. An increased reactivity of the vago-insulin system was unlikely since our previous investigations had suggested that this system is very stable under various experimental conditions.[8] A decreased reactivity of the centers of the sympathetico-adrenal system was likewise improbable since in the acute experiment hypoglycemia elicits a sympathetico-adrenal discharge. (Cannon, McIver and Bliss).[9] Moreover, studies from this laboratory[10] gave evidence of an increased excitability of the medullary sympathetic center in hypogly-

[8] Gellhorn, E., and Feldman, J., *Endocrinology*, 1941, **29**, 467; *Am. J. Physiol.*, 1941, **134**, 603.

[9] Cannon, W. B., McIver, M. A., and Bliss, S. W., *Am. J. Physiol.*, 1924, **69**, 46.

* For details of legend *cf.* Fig. 1.

cemia. However, it was found by Gellhorn and Packer[11] that prolonged anoxia abolished the glycogenolytic action of adrenalin and earlier studies from this and other laboratories showed the close relationship between anoxia and hypoglycemia.[12] Therefore, the question was investigated whether the glycogenolytic action of adrenalin was altered in rats as a result of repeated insulin comas. The results (Table IV) show that the injection of 0.04 mg/kg adrenalin i.p. raises the blood sugar 65% in rats under control conditions. After they had been subjected to 12 insulin comas this dose of adrenalin failed to raise the blood sugar significantly. However, the reaction was restored to a great extent after a rest period of 30 days.

Since, as previously noted, repeated insulin comas raise the initial blood sugar level, the effect of this factor was likewise studied by testing the action of adrenalin on blood sugar on rats which had been fasting for 4 and 22 hours respectively. The results show clearly that a considerable variation in the initial blood sugar fails to alter the glycogenolytic action of adrenalin.

Finally, a similar series was performed on rats subjected to repeated electroshock. Table IV shows that repeated convulsions do not alter the hyperglycemic effect of adrenalin; consequently the increased hyperglycemic response to the anoxia test after repeated electroshocks is not due to an altered reactivity of the liver to adrenalin, and therefore appears to be due to an increased secretion of this hormone.

It is planned to repeat these experiments under conditions which permit a more direct determination of the reactivity of the autonomic centers by the assay of the blood for adrenalin.

Summary and Conclusions. The action of a standard anoxia test involving 5 periods of 25 minutes each at 280 mm Hg was used as a method to evaluate the responsiveness of the centers of the sympathetico-adrenal and vago-insulin systems on the basis of blood sugar determinations. It was found that repeated convulsions induced electrically, increased greatly the hyperglycemic response of rats to the standard test. This is interpreted as a sign of increased reactivity of the sympathetico-adrenal system and is accompanied by a marked dominance of this system as compared to the vago-insulin system. Similar results were obtained after frequent exposures to prolonged anoxia. However, repeated insulin comas failed to bring about this shift and showed actually a diminished or no hyperglycemic response to the standard anoxia test. It was shown that in these rats the administration of adrenalin failed to induce glycogenolysis, which explains the reduction in the hyperglycemic response. Whether the reactivity of the centers of the sympathetico-adrenal systems is increased, as it seems to be by prolonged anoxia and electroshock, will be determined in another investigation by more direct methods. The effects described in this paper were reversible after an interval of 2 to 5 weeks. The work on anoxia and electroshock lends support to the idea that procedures similar to those used in the "shock therapy" of mental disease increase the reactivity of the centers of the sympathetico-adrenal system.

[10] Gellhorn, E., Ingraham, R. C., and Moldavsky, *J. Neurophysiol.*, 1938, **1**, 301; Ingraham, R. C., and Gellhorn, E., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 315; Gellhorn, E., Kieley, W. F., and Hamilton, S. L., *Am. J. Physiol.*, 1940, **130**, 256.

[11] Gellhorn, E., and Packer, A., *Am. J. Physiol.*, 1940, **129**, 610.

[12] *Cf.* Gellhorn, E., *Autonomic Regulations*, New York, Interscience Publishers, 1943.

16397 P

Injection of Mouse Adapted and Egg Adapted Poliomyelitis-like Virus into White Rats.

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During the past year we have cultivated 3 strains of mouse poliomyelitis virus in embryonated eggs. These are a highly mouse-virulent variant derived from the SK strain, the MM strain, and a highly mouse virulent variant derived from the Lansing strain. These were obtained from Dr. C. W. Jungeblut. At the same time we were not successful in cultivating the well known mouse adapted Lansing strain (obtained from Dr. Charles Armstrong) in embryonated eggs. Results of our studies on the immunology of these strains have recently been summarized.[1]

In experimental tests of host susceptibility of the highly virulent mouse strains (*i.e.* intracerebral doses of 0.03 cc of dilutions of 10^{-5} to 10^{-6} being fatal to mice), we injected a group of white rats of about 100 g weight each intracerebrally with 0.1 cc doses of 10^{-1} dilution of mouse brain SK virus. At one week after injection these rats appeared normal with the exception of slight loss of weight. They were sacrificed at this time and the brains removed for virus titrations intracerebrally in mice. Duplicate mice which received 0.03 cc of virus dilutions of 10^{-1} to 10^{-5} inclusive, and also one of 2 mice on a dilution of 10^{-6} died in either 3 or 4 days. A single mouse on a dilution of 10^{-6} survived. These results indicated that while rats exhibited practically no symptoms on first attempted passage of the mouse adapted high SK strain, their brains contained this virus in top titer. Top titers are also obtained by inoculating 0.1 cc of all decimal dilutions of virus up to 10^{-5}. Daily increase of virus is demonstrable and easiest to show following doses of 10^{-3}, 10^{-4} and 10^{-5}. This phenomenon resembles that observed by Hirst[2] when egg adapted strains of influenza virus are passed for the first time in mice which remain asymptomatic although their lungs may contain influenza virus as high in titer as is ever obtained.

In a second experiment we injected strain MM into 6 rats using doses of 0.1 cc of 10^{-1} dilution of mouse brain virus intracerebrally. One week after injection 3 rats were sacrificed for virus titration. A pair of mice receiving 0.03 cc of a single dilution of 10^{-4} of this rat brain virus both died within 3 days. No other mice were tested. The three remaining rats appeared normal 3 weeks after injection, and at this time were bled for tests of neutralizing antibody. The results are shown in Table I and indicate that the inapparent infection resulted in the production of rather strong neutralizing antibody when tested as has been described.[1]

In a third experiment we used virus strain MM in the shape of embryonated egg passage 4. Intracerebral doses of 0.1 cc of a 10^{-1} dilution of the chick embryo virus were injected intracerebrally into rats of about 100 g weight each. Five days after inoculation, part of the groups was sacrificed for virus which titered 10^{-4} intracerebrally in mice. (Incidentally 3 of these rats which were saved appeared healthy and normal one month after inoculation). The MM virus obtained from the sacrificed rats was used for immunization of 2 rabbits. These animals received 2.0 cc of 10^{-1} dilution of active virus intradermally twice weekly, and were bled one week after the tenth and final injection. The rabbit antiserum was then used in neutralization tests in mice against the highly mouse virulent SK virus, MM virus, and the highly mouse virulent Lansing strain. The results of these tests are shown in Table II. It is apparent that rabbit MM antiserum prepared by in-

[1] Powell, H. M., and Jamieson, W. A., *Jour. Inf. Dis.*, in press.

[2] Hirst, G. K., *J. Exp. Med.*, 1947, **86**, 357.

TABLE I.
Neutralization Tests in Mice of Serum from Rats Bled 3 Weeks After Intracerebral Injection of MM Virus.

Dilutions of MM mouse brain virus	Serum used for neutralization test					
	Immune serum			Normal serum		
	i.c.	i.p.	i.n.	i.c.	i.p.	i.n.
10-1	3,3	S,S	6,11	3,3	3,3	4,4
10-3	3,3	S,S	S,S	3,3	4,4	5,6
10-5	S,S	S,S	S,S	3,4	6,S	S,S
10-6	S,S	S,S	S,S	7,S	S,S	S,S

Legend: i.c., i.p., and i.n. indicate respectively intracerebrally, intraperitoneally, and intranasally. Each figure indicates day of death of one mouse. S indicates survival 14 days.

jecting rabbits with rat brain MM virus from asymptomatic rats brings about neutralization of the homologous MM virus and also the other two strains, namely high SK and high Lansing.

In further neutralization tests of this rabbit MM antiserum against the well known "low" Lansing virus, we observed no neutralization whatever. These tests were done by methods indicated in Table III of reference 1, and 10 mice were used on the immune serum plus virus mixture, and 10 mice were used on the normal serum plus virus mixture. At the end of 30 days 9 mice in the immune serum group and 8 mice in the normal serum group had died. The other mice survived.

Conclusions. 1. Strains of highly mouse virulent poliomyelitis virus as transmitted in mice or in embryonated eggs may be transmitted to rats without symptoms but with the development of virus of high titer in the brain in the first passage.

2. Such rat brain virus may be used to immunize rabbits effectively, and the antiserum so obtained neutralizes homologous and heterologous strains of the "high" variety, namely MM, high SK, and high Lansing.

3. The above antiserum does not neutralize the well known "low" Lansing strain of poliomyelitis virus.

TABLE II.
Neutralization Tests in Mice of Serum from Rabbits Immunized with Active Rat Brain MM Virus from Asymptomatic Rats.

Mouse brain virus used	Dilutions of virus	Serum used for neutralization					
		Immune serum			Normal serum		
		i.c.	i.p.	i.n.	i.c.	i.p.	i.n.
High SK	10-1	0	0	0	0	0	0
	10-3	3,S	S,S	S,S	3,3	3,4	4,4
	10-5	S,S	S,S	S,S	3,3	4,4	7,9
	10-7	S,S	S,S	S,S	S,S	0	0
MM	10-1	S,S	S,S	S,S	2,2	3,3	6,6
	10-3	S,S	S,S	S,S	3,3	4,4	7,S
	10-5	S,S	S,S	S,S	4,4	4,S	S,S
	10-7	S,S	S,S	S,S	S,S	S,S	S,S
High Lansing	10-1	3,6	S,S	S,S	2,2	3,3	3,3
	10-3	S,S	S,S	S,S	3,3	4,5	S,S
	10-5	S,S	S,S	S,S	3,3	6,S	6,S
	10-7	S,S	S,S	S,S	4,5	S,S	S,S

Legend: i.c., i.p., and i.n. indicate intracerebrally, intraperitoneally, and intranasally respectively. Each figure indicates day of death of one mouse. S indicates survival 14 days. 0 indicates not done.

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The Precipitation of Latent *Herpes simplex* Encephalitis by Anaphylactic Shock.*

Robert A. Good and Berry Campbell.

From the Department of Anatomy, University of Minnesota.

In a preliminary study[1] we found that anaphylactic shock caused the precipitation of *Herpes simplex* encephalomyelitis from the latent state in a rabbit. The results here reported represent subsequent studies of this phenomenon.

It has long been recognized that Herpes infection in man is frequently recurrent in character and that exacerbations may be produced by a number of stimuli including upper respiratory infections, fever, emotional stress, menstruation, mechanical irritation, heat, exposure to ultra-violet light, and digestive disturbances. The general censensus is that these diverse stimuli activate a latent infection.[2] Levaditi, Harvier, and Nicolau[3] demonstrated Herpes virus in the saliva of normal people. Flexner and Amoss[4] obtained this virus from the cerebrospinal fluid of a luetic patient who was not suffering from Herpes and who gave no history of recent herpetic infection. Bastai and Busacca[5] found Herpes in the cerebrospinal fluid between infections in patients who were subject to recurrent cold sores. Perdrau[6,7] presented data which might be interpreted as evidence of latent Herpes simplex infection in rabbits when he showed that isolation of this virus from the brains of fatal human cases of *Encephalitis lethargica* could be facilitated by mixing the human brain suspensions with brain suspensions of rabbits actively immunized against Herpes. Da Fano and Perdrau[8] and Perdrau[7,9] showed that occasionally herpetic encephalomyelitis in rabbits exhibits a prolonged smouldering course or shows sudden spontaneous exacerbations as long as 6 months after inoculation. Perdrau[7] showed that this indolent type of disease of rabbits could be produced with increased frequency if the inoculated rabbits had first been partially immunized against Herpes.

That the capacity to produce latent infection is not a peculiarity of the Herpes virus has been shown by a large number of clinical and experimental observations demonstrating that many viruses possess such properties. Traub,[10] Skoglund and Baker,[11] MacCallum and Findlay,[12] Traub[13] and Leichenger, Milzer, and Lack,[14] have shown that the virus of lymphocytic choriomeningitis may have latent and intermittently active phases in both animals and man. Similarly, latent infections have been demonstrated to exist with a large number of viruses: infectious anemia virus of horses,[15] foot and mouth disease virus

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1 Good, R. A., and Campbell, B., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 305.

2 Andrewes, C. H., *Proc. Roy. Soc. Med.*, 1940, **33**, 75.

3 Levaditi, C., Harvier, P., and Nicolau, S., *Ann. Inst. Past.*, 1922, **36**, 63.

4 Flexner, S., and Amoss, H., *J. Exp. Med.*, 1925, **41**, 233.

5 Bastai, P., and Busacca, A., *Klin. Wchnschr.*, 1924, **3**, 147.

6-7 Perdrau, J. R., *Brit. J. Exp. Path.*, 1925, **6**, 41, 123.

8 Da Fano, C., and Perdrau, J. R., *J. Path. and Bact.*, 1927, **30**, 67.

9 Perdrau, J. R., *J. Path. and Bact.*, 1938, **47**, 447.

10 Traub, E., *J. Exp. Med.*, 1938, **68**, 229.

11 Skoglund, J. D., and Baker, A. B., *Arch. Neurol. and Psychiat.*, 1939, **42**, 507.

12 MacCallum, F. O., and Findlay, G. M., *Lancet*, 1939, **1**, 1370.

13 Traub, E., *J. Exp. Med.*, 1939, **69**, 801.

14 Leichenger, H., Milzer, A., and Lack, H., *J. A. M. A.*, 1940, **115**, 436.

15 Schalk, A., and Roderick, C. N., *Bull. N. Dak. Agric. Exp. Sta.*, 1923, No. 168, 3.

in cattle,[16] viruses of rinderpest in cattle, hogcholera in swine, fowl pox and laryngotracheitis in hens,[17] Theiler's encephalomyelitis virus in mice,[18] psittacosis virus in birds,[19,20,21] mouse pneumonitis virus,[22,23] pneumonia virus of mice,[24,25] St. Louis encephalitis virus in mice,[26] parotid gland virus of guinea pigs,[27] vaccinia virus in rabbits,[28,29] and perhaps mammary cancer virus in mice.[30,31,32] Furthermore, it may be as Andrewes[2] suggests, that the antibody response to some viruses such as influenza, yellow fever, and poliomyelitis in the absence of clinical disease is due to temporary latent infections.

Kirschbaum[33] and Hoff and Silberstein[34] perhaps by activating a latent infection produced a transmissible encephalitis in dogs by hepatic artery ligation or Eck fistula production. Perdrau[9] employing a number of unrelated devices including subcutaneous guanadine-HCl injections, local brain trauma, and injection of large amounts of inactivated virus was unable to activate latent Herpes and Vaccinia infections, but succeeded in producing the symptomatology and typical pathology of Borna disease in rabbits 18 months after the original infection. Andrewes and Miller,[35] and Rivers and Tillet[36,37] were able to precipitate apparently latent virus III testicular infections in rabbits. McIntosh[38] was able to precipitate sarcoma virus infection by injecting tar into the connective tissue of infected animals. In plants, Bawden,[39] Grainger,[40] Best,[41] Walker and Larson,[42] Shapovalov and Lesley[43] have shown that manipulation of environmental factors, particularly temperature and light intensity, can bring about activation of latent virus infections. Traub[13] has precipitated lymphocytic choriomeningitis infection from the latent state in mice by injecting nutrient broth intracerebrally and Shope has precipitated latent influenza virus infection in swine.[45–49]

16 Waldmann, O., Trautwein, K., and Pyl, G., *Zentralbl. f. Bakt.*, 1931, **121**, 19.

17 Gibbs, C. S., *J. Infect. Dis.*, 1933, **53**, 169.

18 Theiler, M., *Science*, 1934, **80**, 122.

19 Bedson, S. P., *Brit. J. Exp. Path.*, 1938, **19**, 353.

20 Bedson, S. P., *Proc. Roy. Soc. Med.*, 1937-38, **31**, 59.

21 Meyer, K. S., and Eddie, B., *Klin. Wchnschr.*, 1934, **13**, 865.

22 Dochez, A. B., Mills, K. C., and Mulliken, PROC. SOC. EXP. BIOL. AND MED., 1933, **36**, 683.

23 Gordan, F. B., Freeman, G., and Clampet, J. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 450.

24 Horsfall, F. L., and Hahn, R. G., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 684.

25 Horsfall, F. L., and Hahn, R. G., *J. Exp. Med.*, 1940, **71**, 391.

26 Webster, L. T., *J. Exp. Med.*, 1938, **68**, 11.

27 Jordan, E. O., and Burrows, W., *A Textbook of Bacteriology*, W. B. Saunders, Philadelphia and London, 1945, p. 798.

28 Douglas, S. R., Smith, W., and Price, L. R. W., *J. Path. and Bact.*, 1929, **32**, 99.

29 Olitsky, P. K., and Long, P. H., *J. Exp. Med.*, 1929, **1**, 263.

30 Bittner, J. J., *Am. J. Cancer*, 1939, **35**, 90.

31 Green, R. G., *Minn. Med.*, 1946, **29**, 277.

32 Green, R. G., Mossey, M. M., and Bittner, J. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 362.

33 Kirschbaum, W., *Z. ges. Neurol. u. Psychiat.*, 1923, **87**, 50.

34 Hoff, H., and Silberstein, F., *Z. ges. exp. Med.*, 1924-25, **44**, 268.

35 Andrewes, C. H., and Miller, C. P., *J. Exp. Med.*, 1924, **40**, 789.

36 Rivers, T. M., and Tillet, W. S., *J. Exp. Med.*, 1923, **38**, 673.

37 Rivers, T. M., and Tillet, W. S., *J. Exp. Med.*, 1924, **39**, 777.

38 McIntosh, J., *Brit. J. Exp. Path.*, 1933, **14**, 422.

39 Shapovalov, M., and Lesley, J. W., *Phytopath.*, 1931, **21**, 83.

40 Bawden, F. C., *Plant Viruses and Virus Diseases*, 1943, Chronica Botanica Co., Waltham, Mass., U.S.A., p. 18.

41 Grainger, J., *Nature*, 1938, **137**, 31.

42 Best, R. J., *Aust. J. Exp. Biol. and Med.*, 1936, **14**, 223.

43 Walker, J. C., and Larson, R. H., *J. Agr. Res.*, 1939, **59**, 259.

44 Good, R. A., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 360.

45 Shope, R. E., *J. Exp. Med.*, 1941, **74**, 49.

46 Shope, R. E., *J. Exp. Med.*, 1941, **74**, 41.

47 Shope, R. E., *J. Exp. Med.*, 1943, **77**, 127.

48 Shope, R. E., *J. Exp. Med.*, 1943, **77**, 111.

49 Shope, R. E., *Medicine*, 1944, **23**, 415.

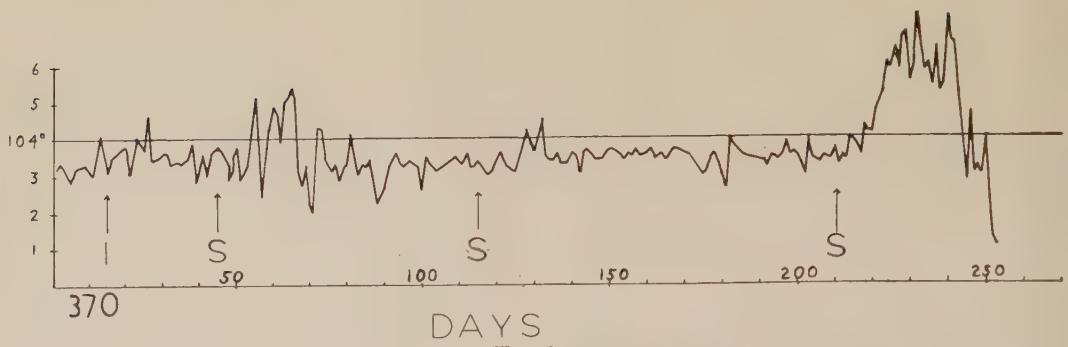

FIG. 1.

Temperature curve of Rabbit No. 370. Three instances of precipitation of *Herpes simplex* infection following anaphylactic shock. I—inoculation of H.F. virus. S—administration of anaphylactic shock. *—virus recovered 9 months after initial infection.

Materials and Methods. This experiment was performed on young adult albino rabbits which were sensitized by the administration of egg white on alternate days in the following dosages: 1 cc i.v.; .5 cc i.v.; 1 cc i.m. The rabbits were then inoculated into the quadriceps muscle group with .5-1 cc of a 10^{-1} suspension of mouse brain from mice which had died with *Herpes simplex* encephalitis.

Inoculation of rabbits in this way produces 5 courses of disease. 1. Somewhat less than 50% of inoculated rabbits develop fever, acute ascending myelitis; encephalitis, meningitis and death in a 3 to 4 week period.

2. Some rabbits develop acute disease with high fever, myelitis, encephalitis, and meningitis, but survive and apparently completely recover from the infection.

3. Some develop a mild form of the disease with minimal symptoms of encephalomyelitis and early recovery.

4. A few develop chronic, smouldering progressive encephalomyelitis with lowgrade fever and flare-ups of increased activity.

5. Some rabbits show no demonstrable infection.

From a large group of rabbits inoculated into the quadriceps muscle as described those showing courses 2, 3, 5 were selected for further study. Daily temperatures were recorded and each rabbit was checked regularly for the presence of symptoms. After evidence of the acute infection had disappeared and the rabbits had been well for from 1 to 3 months each was subjected to anaphylactic shock by

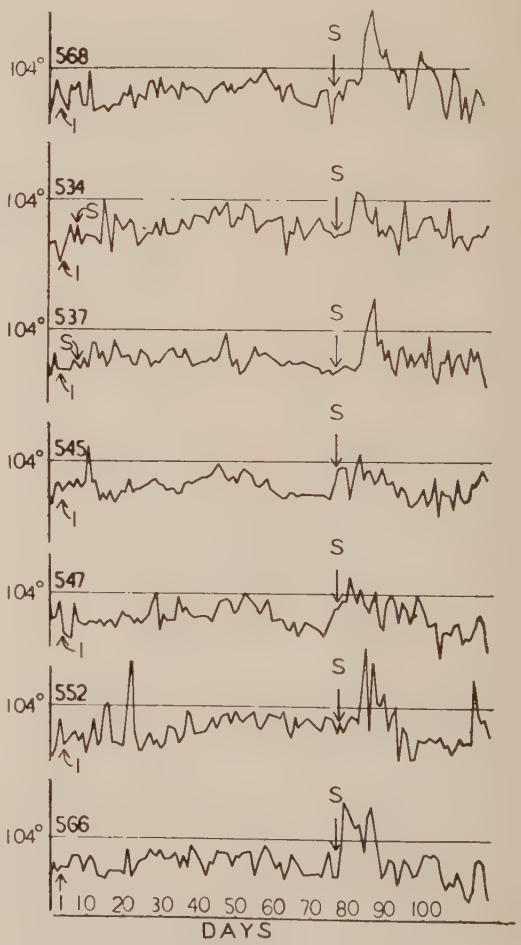

FIG. 2.

Temperature curves from 6 rabbits. Each curve shows an instance of precipitation of latent Herpes infection following anaphylactic shock. I—inoculation with *Herpes simplex* virus. S—administration of anaphylactic shock.

TABLE I.
Precipitation of Latent *Herpes simplex* Infection in Rabbits.

No.	No. of precipitations	Date of original virus inoc.	Date of onset of precipitation	Degree of shock	Incubation period days	Fever to	Manifestion of disease
531	1	10-1-46	2- 1-47	mild	5	105.4	encephalitis, myelitis, paralysis, death.
534	2	”	12-23-46	”	5	104.4	” meningitis.
			2- 1-47	”	6	105.2	” ”
537	1	”	12-26-46	moderate	8	105.8	”
545	1	”	12-23-46	mild	5	104.4	”
547	1	”	12-21-46	moderate	3	104.9	” meningitis, myelitis, death.
548	1	”	1-22-47	”	2		
552	1	”	12-24-46	mild	5	106.4	” ”
562	1	”	1-31-47	no apparent shock	6	107.2	” prolonged illness.
563	1	”	1-30-47	mild	7	105.3	” meningitis.
568	1	”	12-25-46	”	7	107.2	” myelitis
569	1	”	12-22-46	”	5	106.4	” ” prolonged C.N.S. illness, death.
566	1	”	12-19-46	”	2	106.0	signs of encephalitis.
679	1	12-18-46	5- 1-47	no apparent shock	14	104.8	encephalitis, myelitis.
681	1	”	4-22-47	mild	6	105.4	” death.
370	3	12-22-45	2- 7-46	moderate	9	104.6	” myelitis, meningitis.
			4-22-46	mild	7	104.6	”
			7-10-46	severe	8	107.4	” myelitis, death.
126	1	7-30-44	2-13-45	”	5	106.2	” meningitis, death.

the intravenous injection of .2 to .6 cc of egg white. The ensuing shocks were graded mild, moderate, or severe.

Results. Fig. 1 shows the temperature curve of an illustrative animal.

Fig. 2 shows the temperature curves of a representative series.

The findings of these experiments are summarized in Tables I and II. Encephalomyelitides were precipitated from the latent state 19 times in 16 rabbits. The symptoms of encephalitis included disorientation, gnashing of teeth, nystagmus, muscular twitchings, drooling of saliva, and vestibular fits. Opisthotony was considered to be evidence of meningitis and flaccid paralysis and diminished or absent deep reflexes in the extremities as evidence of myelitis. The anaphylactic shocks were not followed immediately by activity of the infection, but in each case an incubation period varying between 2 and 14 days separated the anaphylactic shock from the exacerbation. The mean incubation period

TABLE II.
Incidence of Precipitation of *Herpes simplex* Encephalitis Following Anaphylactic Shock.

1.	No. of rabbits with possible latent infection studied	44
2.	No. of anaphylactic shocks administered	52
3.	No. of rabbits showing the precipitation phenomenon	16
4.	No. of precipitation infections noted	19
5.	No. of apparently spontaneous exacerbations noted during period of study	4
6.	No. of times anaphylactic shock failed to induce precipitation of *Herpes simplex*	28
7.	No. of rabbits showing questionable precipitation following anaphylactic shock	5

was 6 days and the most commonly occurring one was 5 days. The spontaneous and induced exacerbations were symptomatologically alike, and in fatal cases from both groups virus was recovered from the central nervous system which was identical to the Herpes virus inoculated several months previously. In the 5 instances described as questionable precipitations in Table II the active infection was too far removed from the anaphylactic shock to be unequivocally attributed to it in 2 cases and the evidence of activity was questionable in 3.

Table I contains some evidence that the severity of the shock shows little correlation with the incidence of activation of latent infection. According to the criteria used 2 rabbits demonstrating no shock following reinjection of egg white showed the precipitation phenomenon and several showed successful activation with mild shock.

In order to compare the incidence of precipitation due to anaphylactic shock with that of spontaneous activation the following formulae were used.

$$I_1 = \frac{P}{T_1} = \frac{19}{728} = \frac{26.4}{1000} \text{ rabbit days of exposure}$$

$$I_2 = \frac{S}{T_2} = \frac{4}{7934} = \frac{.5}{1000} \text{ rabbit days of exposure}$$

Where I_1 = incidence of precipitation infections, P = number of precipitation infections, T_1 = number of anaphylactic shocks administered $\times$ 14 (arbitrary number of days of anaphylactic shock influence), I_2 = incidence of spontaneous infection, S = number of spontaneous infections; T_2 = total rabbit days of the experiment $-T_1$.

Herpes simplex virus was recovered from the central nervous system of rabbits of this series following fatal precipitation infection, during periods of quiescence after precipitations, following fatal spontaneous exacerbations, and during chronic smouldering encephalitis. At autopsy the brains and spinal cords of the rabbits were removed under aseptic precautions, placed in 50% glycerine in normal saline buffered to pH 7.4, and stored in the icebox from 3 days to 3 weeks. Pools were made of the central nervous tissues of each rabbit, ground with mortar and pestle with normal saline to prepare a 10% suspension of rabbit brain and inoculated intracerebrally into young adult mice. In the cases in which the mice developed fatal encephalitis several passages through mice were accomplished and then further studies were carried out to identify the virus by its biological and immunological properties. These studies included titration in mice, corneal inoculation in rabbits, pathological study of brains of corneally infected rabbits and neutralization studies with immune rabbit serum and pooled human serum.

Discussion. These data raise questions concerning the nature of the disease observed during the periods of exacerbation. Is it a renewal of the same disease which was re-

sponsible for the original illness, or without the renewal of the activity of the infectious agent does the anaphylactic shock merely activate old lesions? Is the exacerbation a true herpetic encephalitis, or is it merely a flare-up of an old inflammatory locus due to some non-specific stimulus? The evidence available at the moment indicates that we are dealing with a renewal of the essential disease process. From the point of view of the symptomatology, it may be observed that the exacerbations frequently show more severe and more widespread evidence of disease than do the initial infections. This would indicate enlargement of the affected area in the nervous system. Even more convincing is the evidence already reported[44] of recovery of *Herpes simplex* virus from the nervous system of these rabbits as long as 9 months after the initial infection. The virus is to be found in the nervous system not only in the active stages of the disease, but also during quiescent remissions. To interpret these results in terms of the relationship of antigen-antibody reactions to the dynamics of infection is speculative, but hypotheses based on this line of thinking are intriguing. It seems that these rabbits have become carriers by overcoming the initial infection yet continuing to harbor virus in their tissues. That anaphylactic shock results in a breakdown of this symbiosis in which the virus is present in the central nervous system without signs of disease may indicate that immunity to the virus suffers a temporary lapse during or following the anaphylactic shock which allows the virus once again to express its virulence. To claim that anaphylactic shock is in any way a specific means of precipitating latent neurotropic virus infection is not intended, but whatever might be the alteration in the immune or physiologic state of the animals which is responsible for disruption of the established host-parasite balance, it is effectively produced by anaphylactic shock. Although the recently recognized encephalitis following pertussis immunization shows a striking parallelism to this phenomenon, we are unaware of the demonstration of identical disturbances in recognized human disease. Careful elucidations of the factors involved in the largely unexplored realm of post-infectious and undiagnosed encephalitides commonly occurring in childhood, and in exacerbations of symptomatology in the post-encephalitic patients might reveal mechanisms in common with the experimental disease.

Summary. 1. Evidence that virus disease may develop a latent state is reviewed with an account of the reports of successful activations of latent infections.

2. Nineteen instances of precipitation of latent herpetic infection in rabbits by anaphylactic shock are presented.

3. Recovery of *Herpes simplex* virus from rabbit central nervous system during periods of (a) quiescence, (b) chronic encephalitis, (c) spontaneous exacerbations, and (d) anaphylactically induced precipitations of encephalitis is reported.

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Invasion by *Shigella sonnei* of Tissues of Mice Following Gavage with Viable Shigella.*

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In a previous publication[1] we reported that white mice did not show any signs of infection but did develop a considerable degree of active immunity against intraabdominal chal-

* Supported in part by a grant from the U. S. Public Health Service.

1 Cooper, Merlin L., and Keller, Helen M., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 442.

TABLE I.
Number of Mice in Whom Viable Shigella Were Found Outside the Gastrointestinal Tract Following Gavage with Viable *Shigella sonnei.*

Source of cultures	Incidence of positive cultures Time of culture after gavage			
	15-30 min.	45-120 min.	24 hrs	Total
Spleen	14/29*	6/22	1/20	21/71
Liver	15/29	6/22	1/20	22/71
Kidney	13/29	7/22	1/20	21/71
Mes. Gl.	27/29	15/22	1/20	43/71
Lung	22/29	10/22	2/20	34/71
Heart	18/29	6/22	1/20	25/71
Tail	2/29	0/22	1/20	3/71

* Numerator = No. of mice with positive cultures. Denominator = No. of mice cultured.

lenge following administration by gavage of viable *Shigella sonnei.*

This evidence of antigenic stimulus raised the question whether soluble antigen was absorbed from the gastrointestinal tract or whether the Shigella, still viable, might invade or be transported from the gastrointestinal tract into various tissues of the mice.

Data are presented in this report showing the results when cultures were made of various tissues of mice sacrificed at intervals following gavage with viable *Shigella sonnei.* Tail blood was also cultured.

Methods. The strain of *Shigella sonnei* (Ch) used in these experiments had been isolated by us several years ago from the stool of a patient (Ch) acutely ill with bacillary dysentery in the Children's Hospital. It has remained highly mouse virulent. Brain heart infusion (Difco) cultures of this strain of Shigella 18 to 20 hours of age were used for gavaging. The viable bacterial counts were usually 1.2 billion organisms per ml.

For gavaging a 2 ml luer-lock syringe was used with a blunt 18 gauge needle 2 inches in length. The needle was passed over the tongue and down the esophagus into the stomach where 1 ml of culture was deposited.

At intervals after gavage, tail blood was obtained for culture. The distal half of the tail was painted with tincture of mercresin for 3 minutes. The mercresin was removed with an alcohol sponge and the tail was dried with a sterile dry sponge. The end of the tail was clipped with sterile scissors and 2 or 3 drops of blood obtained and cultured in brain heart infusion (Difco). The mouse was then sacrificed by inhalation of ether. The abdomen and thorax were opened under sterile conditions and the spleen, kidney, central mesenteric gland, part of the liver, part of the lungs and finally the heart were each removed in the order mentioned with separate sterile instruments. Sterile gauze was packed around organs to absorb local bleeding. Each specimen of tissue was cultured in separate tubes of brain heart infusion (Difco). All positive cultures were studied and the organism present identified.

Data in Table I show the number of mice in which *Shigella sonnei* were found in different tissues outside the gastrointestinal tract at intervals after giving them by gavage 1.2 billion viable *Shigella sonnei.* Of 29 mice sacrificed and cultured 15 to 30 minutes after gavage, *Shigella sonnei* were cultured from: the central mesenteric glands of 27 mice; the lungs of 22; the hearts of 18; the livers of 15; the spleens of 14; the kidneys of 13, and the tail blood of only 2 mice. Of 22 mice sacrificed and cultured 45 minutes to 2 hours after gavage, *Shigella sonnei* were cultured from: the mesenteric glands of 15 mice; the lungs of 10; the kidneys of 7; and the hearts, livers and spleens of 6 mice. All tail blood cultures from this group of 22 mice were sterile. Cultures from tissues of mice sacrificed 24 hours after gavage were usually negative. Of a group of 20 mice sacrificed at this time positive cultures were obtained from only 2 animals.

The highest incidence of positive cultures came from the central mesenteric glands, a finding to be expected if the organisms were

TABLE II.

Cultures of Mouse Tissues Removed Within 5 to 10 Minutes After Giving Various Numbers of Viable *Shigella sonnei* by Gavage.

Mouse wt, g	Dose of culture, millions	Spleen	Liver	Kidney	Mesenteric gland	Lung	Heart	Tail blood
8	1200	+	+	+	+	+	+	—
8	1200	+	+	+	+	+	+	+
8	1200	—	+	+	+	+	+	—
8	1200	+	+	+	+	+	+	—
8	1200	—	—	—	+	+	+	—
8	1200	—	—	+	+	+	+	
8	750	+	+	+	+	+	+	
10	300	+	+	+	+	+	+	
10	150	+	+	+	+	+	+	
10	150	—	—	—	—	+	—	
10	15	—	+	+	+	+	+	
9	13	—	—	—	+	—	—	
9	1.5	—	—	—	—	—	—	
10	1.3	—	—	—	+	—	—	
11	0.15	—	—	—	—	—	—	
10	0.01	—	—	—	+	—	+	

transported from the intestinal tract by way of the lymphatic system.

The occurrence of the second highest incidence of positive cultures from lung tissue raises the question of the possibility of aspiration of culture at the time of gavage. This possibility is difficult of exclude, but if a tightly fitting syringe is used, there is no evidence of leakage from the syringe during the process of gavaging. The volume of culture deposited in the stomach was not large enough to overflow up the esophagus and into the lungs.

The occurrence of the third highest incidence of positive cultures from the heart indicates that the blood is another means of their transportation. With positive heart cultures the majority of other tissues cultured might be expected to show, as indeed they did show, the presence of the organisms. Of a group of 23 mice sacrificed 15 to 60 minutes after gavage, all with positive heart cultures, there were 22 mice with positive mesenteric gland cultures; 21 with positive lung cultures; 19 with positive liver cultures; 17 with positive spleen cultures; 15 with positive kidney cultures and 2 with positive tail blood cultures. On the other hand, if heart cultures were negative, fewer cultures from other tissues might be expected to be positive. This was found to be the case, except for cultures of the mesenteric glands, all of which showed the presence of *Shigella sonnei*. For example, of a group of 19 mice sacrificed, 15 to 60 minutes after gavage, all with negative heart cultures, the Shigella were found in the mesenteric glands of all 19 mice; in the lungs of 8; in the kidneys of 5 and in the spleens and livers of 3 mice.

The role of the reticuloendothelial cells in removing the Shigella from the circulation and tissues is most likely great. However, examination of tissue sections from some of these mice failed to show any recognizable activity of the cells of this system. We are indebted to Dr. Ralph J. Johansmann for these studies.

Influence of the number of Shigella given by gavage on their invasion outside the gastrointestinal tract. Data in Table II show the results of culturing tissues removed from mice weighing 8 to 11 g within 5 to 10 minutes after they had been given various numbers of viable *Shigella sonnei* (Ch) by gavage. It is observed that doses above 13 million organisms usually resulted in positive cultures while doses of 13 million and less gave very few positive cultures. When the same type of experiment was done with mice weighing 17 to 18 g, doses of 150 million viable Shigella were the minimal number above which most cultures of tissues were positive and below which few cultures were positive. These data indicate not only that the number of viable

Shigella given by gavage was a factor influencing invasion from the gastrointestinal tract, but that within 5 minutes after their administration by gavage they could be cultured from tissues of the mice.

Summary. *Shigella sonnei,* when given by gavage to white mice, invaded beyond the walls of the gastrointestinal tract; were found within 5 to 30 minutes in cultures of spleens, livers, kidneys, central mesenteric glands, lungs and hearts of the majority of mice; and were seldom cultured from these tissues after 2 hours from the time of gavage. The Shigella were found most often in cultures of the central mesenteric glands and least often in cultures of tail blood.

The number of viable Shigella and the weight of the mice were factors influencing the invasion by the Shigella from the gastrointestinal tract. The minimal number of viable Shigella which, when given by gavage, invaded beyond the gastrointestinal tract, was 150 million for mice weighing 17 to 18 g and 13 million for mice weighing 8 to 11 g.

16400

Effect of Non-Specific Protein (Aolan) upon Twelve-Hour Nocturnal Gastric Secretion in Man.*

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A recent study[1] demonstrated that nocturnal and 24-hour gastric secretion may diminish in man during the intramuscular injection of large quantities of an enterogastrone concentrate. This decrease is variable in degree, temporary in duration, and often followed by an increased output of acid. Since the concentrate contains a mixture of proteins, the possibility of a non-specific effect was considered. The present study was undertaken to further clarify this question.

Previous investigations of the effect of non-specific proteins upon gastric acidity have dealt with secretion stimulated by various test meals or by histamine. Vanzant and Snell[2] observed, in nine healthy dogs injected intravenously with a killed culture of *B. prodigiosus,* a decrease in the volume and acidity during the febrile period, followed by a more prolonged rise, and 48 hours later by normal values. Meyer, Cohen and Carlson[3] also noted a temporary decrease in gastric acidity during the fever induced by *B. prodigiosus.* The acidity of the fasting contents removed during the febrile period in 6 of 17 patients with peptic ulcer, treated with triple typhoid vaccine or Lilly's type "H" antigen, was unchanged in 2 and decreased in 4; in one case the acidity increased 3-fold several hours after the termination of fever. Following the treatment, there was no constant change in acidity.[2] Most writers have reported a decrease in gastric secretion during fever, although the results vary considerably.[4] Martin[5] found no constant rise or fall in the acidity of patients with peptic ulcer given repeated intramuscular injections of 10 cc of a purified milk preparation (Aolan).

Method of Study. Ten patients, 9 males and 1 female, between the ages of 36 and 61 years, were studied. A duodenal ulcer was present in 9 and a jejunal ulcer in 1 male patient. The 12-hour nocturnal gastric secre-

* This study was supported in part by a grant from the Upjohn Co., Kalamazoo, Mich.

1 Kirsner, J. B., Levin, E., and Palmer, W. L., *Gastroenterology,* 1948, **10**, 256.

2 Vanzant, F. R., and Snell, A. M., *J. Clin. Invest.,* 1930, **46**, 768.

3 Meyer, J., Cohen, S. J., and Carlson, A. J., *Arch. Int. Med.,* 1918, **21**, 354.

4 Meyer, J., and Kartoon, L. B., *Arch. Int. Med.,* 1930, **46**, 768.

5 Martin, L., *Arch. Int. Med.,* 1929, **43**, 299.

TABLE I.
Effect of Non-Specific Protein (Aolan) on 12-Hour Nocturnal Gastric Secretion in Man.
(10 Patients.)

Patient	Control period		After Aolan*	
	Vol. (cc)	Output HCl (mg)	Vol. (cc)	Output HCl (mg)
1. E.M. 370374 Jejunal ulcer	1176	3933	737	2518
	1325	4894		
	1108	3044		
2. C.B. 130099† Duod. ulcer	508	1026	664	1158
3. R.B. 160290 Duod. ulcer	784	1287	694	1327
4. A.F. 419242† Duod. ulcer	819	1607	1206	3601
	1341	3882		
5. J.F. 420465 Duod. ulcer	531	855	935	1658
6. E.B. 419277 Duod. ulcer	737	1357	1032	2438
7. F.P. 401669 Duod. ulcer	391	1481	790	2685
8. V.R. 420485 Duod. ulcer	561	1134	1062	2953
9. M.S. 415557 Duod. ulcer	584	1496	1084	3344
	895	2351		
10. J.S. 420271† Duod. ulcer	1297	3417	1523	4289

* 20 cc Aolan intramuscularly at 8:30 P. M., onset of nocturnal period.
† Chills and fever (38.4-38.5°C) during period of observation.

tion was measured from 8:30 P.M. to 8:30 A.M. by the continuous suction technic described previously.[6] After control observations had been obtained for 1 to 3 nights, 20 cc of a sterile lactalbumin solution (Aolan) were injected intragluteally at 8:30 P.M.; the stomach then was aspirated as in the control periods. Four patients developed chills and fever up to 38.5°C several hours after the administration of the preparation. The pain accompanying the injections was similar to that observed with enterogastrone.

Results. The data are tabulated in terms of total volumes and output of hydrochloric acid for the 12-hour nocturnal period (Table I). Gastric secretion was unchanged in 3 cases, diminished slightly in 1 and increased in 6; the rise in 4 of the latter group was nearly 2-fold. The hourly pattern of secretion following the injection of Aolan resembled that of the control periods. Among the 4 patients who developed fever, the output of acid was unaltered in 2 and increased in 2 cases.

Summary. The output of hydrochloric acid in the 12-hour nocturnal gastric secretion of 10 patients with peptic ulcer, following the intramuscular injection of 20 cc of a sterile lactalbumin solution, was unchanged in 3 cases, decreased slightly in 1 and increased in 6 patients. These results suggest that the temporary reduction in gastric secretion occasionally observed during the administration of enterogastrone is not attributable to a non-specific action of the proteins present in the concentrate.

[6] Levin, E., Kirsner, J. B., Palmer, W. L., and Butler, C., *Arch. Surg.*, in press.

16401

Volume of Oil and Route of Administration as Factors Influencing Testosterone Propionate Activity.

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Seminal vesicle weight reaches a maximum in 72 hours following a single injection of testosterone propionate at a dosage of 0.25 mg. Under these circumstances, the androgen can be assayed in the rat since variation in dosage significantly influences the organ weight response.[1] This steroid is usually administered in oil and it seemed pertinent to investigate the volume of oil as a factor influencing the response since sesame oil has been shown not to be inert.[2] Furthermore, Crafts[3] found that sesame oil may prevent the expected effect from small dosages of androsterone in mice whereas Deanesly and Parkes[4] increased the effectiveness of androsterone in the rat by greatly increasing the volume of oil.

The route of administration, too, deserves consideration since subcutaneous and intramuscular injections are commonly used. The intraperitoneal route is reported as both an ineffective[5] and effective[6] mode of administration on repeated injections but the single injection response was not considered.

Immature male rats of the Long-Evans strain and 22-24 days of age were used in these experiments. A standard dose of 0.25 mg of testosterone propionate (Perandren, Ciba)* was used. Each group of animals was caged separately and 72 hours after the single injection the rats were autopsied and the fresh weight of the testes, seminal vesicles (including the coagulating gland) and ventral prostate was obtained.

The administration of a single subcutaneous injection of 0.25 mg of testosterone propionate in either 0.1, 0.4 or 0.8 cc of sesame oil induced similar organ weight changes in comparison with normal and oil injected controls (Table I). The androgen increased seminal vesicle weight more than 300% without regard to volume of oil and had a tendency, although not significantly, to be more effective in the larger volume. The ventral prostates were stimulated to the same degree in each group but the increase in weight was slight in comparison with the seminal vesicles. Testis weight was reduced by the androgen but body weight increased normally.

Fourteen rats received the 0.25 mg dosage of androgen dissolved in 1.6 cc of sesame oil. The steroid was administered subcutaneously in 2 sites on opposite sides of the body and in 0.8 cc amounts, the entire 1.6 cc being given at the same time. Seventy-two hours later seminal vesicle weight average 34.0 mg or "possibly significantly" greater than when smaller volumes and 1 injection were used. Ventral prostate weight averaged slightly greater than in the other groups, being 47.8 mg.

A study of the route of administration revealed that 0.25 mg of androgen in 0.1 cc sesame oil will increase seminal vesicle and ventral prostate weights to the same degree by either subcutaneous or intramuscular injections (Table II). The subcutaneous injections were made under the loose skin area of the dorsal neck aspect; the intramuscular injections were made into the medial thigh

[1] Hays, H. W., and Mathieson, D. R., *Endocrinology*, 1945, **37**, 266.

[2] Tobin, C. E., *J. Lab. and Clin. Med.*, 1944, **29**, 850.

[3] Crafts, R. C., *Endocrinology*, 1942, **31**, 142.

[4] Deanesly, R., and Parkes, A. S., *Lancet*, 1936, **1**, 837.

* Testosterone propionate (Perandren) was generously supplied by Ciba Pharmaceutical Products, Summit, N.J.

[5] Deanesly, R., and Parkes, A. S., *Proc. Roy. Soc.*, 1937, **124**B, 279.

[6] Rubinstein, H. S., *J. Urology*, 1944, **51**, 88.

TABLE I.
Volume of Sesame Oil and Response of Immature Male Rats to Single Subcutaneous Injection of Testosterone Propionate.

No. of rats	Treatment	Body wt		Avg organ wt, mg		
		Start, g	End, g	Testis	Seminal vesicles	Ventral prostate
16	None	47.3	54.1	384 ± 34*	8.2 ± 0.7	33.1 ± 2.1
10	.8 cc oil	42.8	49.8	319 ± 24	8.4 ± 3.5	28.0 ± 1.5
16	.25 mg in 0.1 cc	44.0	51.9	275 ± 14	27.5 ± 1.1	43.0 ± 2.2
14	.25 " " 0.4 "	43.8	51.6	270 ± 10	29.2 ± 1.5	41.8 ± 1.6
14	.25 " " 0.8 "	43.8	53.6	290 ± 13	30.2 ± 1.7	43.7 ± 1.6

$$^{*}\epsilon = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$$

TABLE II.
Route of Administration and Immature Male Rat Response to a Single Injection of Testosterone Propionate.

No. of rats	Treatment	Body wt		Avg organ wt, mg		
		Start, g	End, g	Testis	Seminal vesicles	Ventral prostate
12	None	47.5	54.9	400 ± 42	9.7 ± 0.9	34.9 ± 1.9
13	.25 mg—intrap.	50.6	58.6	424 ± 50	12.2 ± 1.1	39.0 ± 3.0
12	.25 mg—subcu.	45.6	47.8	306 ± 30	29.2 ± 3.0	42.9 ± 3.0
16	.25 mg—intramusc.	46.3	60.7	305 ± 47	30.5 ± 2.2	43.6 ± 3.3

muscles. In contrast to these results, the intraperitoneal route exercised little effect on the seminal vesicles and ventral prostate and no effect on the testis.

Discussion. The response to testosterone propionate was not influenced by the volume of oil. Similar results were obtained with estrogens given in repeated dosages.[7] The volume of oil may, however, be a factor when steroids of low solubility are used or, in fact, may influence less active androgens.[3,4] The prostate responsiveness to androgen is considered as more acute than seminal vesicle responsiveness but certainly the degree of response favors the seminal vesicles following a single injection. The increase in prostate weight in a normal rat would permit little weight differential for assay purposes. Furthermore, Steadman and Krichesky[8] find that the varied prostatic lobes do not respond equally to testosterone propionate.

Summary. The increase in seminal vesicle and ventral prostate weights in immature male rats following a single subcutaneous injection of 0.25 mg of testosterone propionate is not influenced by the volume of oil (0.1-0.8 cc). Seminal vesicle weight increased to a greater degree than the ventral prostate weight whereas testes weight decreased. Subcutaneous and intramuscular routes of administration increased accessory sexual organ weight equally well in contrast to the intraperitoneal route which exercised little effect following one injection.

7 Pugsley, L. I., and Morrell, C. A., *Endocrinology*, 1943, **33**, 48.

8 Steadman, F. H., and Krichesky, B., *Endocrinology*, 1945, **37**, 89.

16402

Production of Nitrogen Retention in Hypophysectomized Rats by Small Doses of Hypophyseal Growth Hormone.*

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Previous work from this laboratory has demonstrated that the administration of 1 mg per day of pure hypophyseal growth hormone will produce nitrogen retention and weight gain in normal adult female rats[1] and that .01 mg per day will produce weight gain in *ad libitum* fed hypophysectomized rats.[2] This present study was undertaken to determine whether the administration of such small amounts of growth hormone would produce a demonstrable nitrogen retention in hypophysectomized rats.

Methods. Ten male rats of the Long-Evans strain were used as the experimental animals. They were hypophysectomized through the parapharyngeal approach when they were between 58 and 65 days of age. Following hypophysectomy they were maintained in individual metabolism cages and their average *ad libitum* food intake† was determined for 11-13 days. Daily urine collections were started 11-13 days after hypophysectomy and the urine was analyzed for total nitrogen by the Kjeldahl procedure.

During the entire urine collection period each rat was fed daily a weighed amount of diet which was the same or slightly less than the rat's average daily consumption during the previous post-hypophysectomy period. Thus the food consumption of each rat was nearly constant throughout the entire experimental period and was restricted in amount.

Urine was collected for a 10-day control period prior to growth hormone injection and for a 4-day control period after cessation of injection. The growth hormone used was prepared according to the previously published method[2] and was administered twice daily intraperitoneally. It was given at dose levels of .05 mg, .03 mg and .015 mg daily.

Results. The data from the experiment are presented graphically in Fig. 1 and analyzed statistically in Table I. It will be seen that during the growth hormone injection period there was a slight increase in average food intake. This was due to the fact that only occasionally did a rat fail to consume completely the food that was offered. In spite of the slight increase in food consumption, and hence nitrogen intake, there was a decrease in the average urinary output of nitrogen during the administration of growth hormone at all dose levels. The largest dose of growth hormone, 50 γ per day, produced the greatest nitrogen retention, 53 mg per day. The smallest dose of growth hormone, 15 γ per day, produced the least nitrogen retention, 17 mg per day. As demonstrated by Fig. 1, during the injection period of 15 γ of hormone per day the amount of urinary nitrogen excreted the first day was less than the amount excreted the following days. This undoubtedly reflects a continuing effect of the

* Aided by grants from the American Cancer Society (through the National Research Council—Committee on Growth); the U. S. Public Health Service—RG-409; and the Research Board of the University of California.

1 Gordan, G. S., Bennett, L. L., Li, C. H., and Evans, H. M., *Endocrinology*, 1948, **42**, 153.

† The diet fed consisted of ground whole wheat 67.5%, casein 15.0%, whole milk powder 10.0%, NaCl 0.75%, $CaCO_3$ 1.5%, hydrogenated vegetable oil (Crisco or Primex) 5.25%. To each kg of diet were added 3.5 g of Sardilene (fish oil concentrate containing 3000 USP units vitamin A and 400 Chick Units vitamin D per g).

2 Li, C. H., Evans, H. M., and Simpson, M. E., *J. B. C.*, 1945, **159**, 353.

3 Fisher, R. A., *Statistical Methods for Research Workers*, 10th Ed., Edinburgh, London, Oliver and Boyd, 1946.

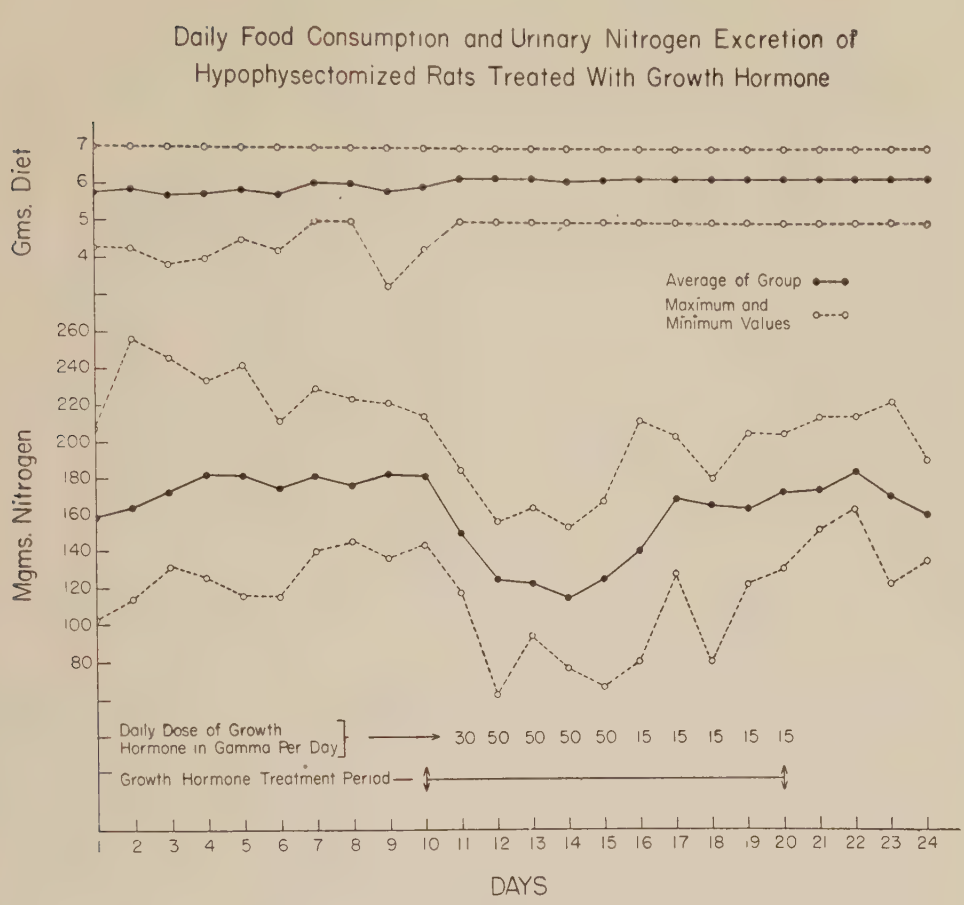

FIG. 1.

previous larger dose. If this day is omitted in calculating the averages the effect of the 15 γ dose is not significant. The intermediate dose, 30 γ per day, produced an intermediate effect. After the cessation of injection the urinary nitrogen excretion returned to its original level.

Conclusion. Growth hormone administration in amounts as small as 30 γ per day produces nitrogen retention in hypophysectomized rats maintained on a constant and restricted food intake.

TABLE I.
Average Daily Food Intake and Urinary Nitrogen Excretion of Hypophysectomized Rats Treated with Growth Hormone.

Daily dose growth hormone	Food intake, g	Urinary nitrogen, mg	p‡
None (pre-injection control)	5.84	176 ± 3.1* (100)†	
.015 mg	5.83	159 ± 4.2 (50)	.01
.030 mg	6.12	151 ± 7.4 (10)	.02
.050 mg	6.16	123 ± 4.3 (40)	.01
None (post-injection control)	6.20	173 ± 3.4 (40)	.70

* Standard deviation of the mean.
† Number of observations in group.
‡ From Fisher's[3] table of t.

16403 P

Dependence of Ovarian Spiral Arteries on the Trophic Action of Estrogens.*

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Patterns of spiral arteries in rabbit[1-3] and human ovaries[4,5] have been described. Although there are differences in the arrangement of these vessels in the two species, homologous parts may be discerned. In the human, the ovarian artery passes along the hilus of the ovary, inosculating with its uterine branch. This may be called the basic branch and compares with the same vessel in the rabbit which lies wholly outside the ovary. There are numerous short tortuous vessels arising primarily from this basic vessel in the human and these primary branches compare with the single (or sometimes double[2]) ramus ovaricus in the rabbit. In the human, each primary branch normally gives rise to a number of still smaller helical blood vessels that pass in the folds of the posterior duplicature of the broad ligament as this envelopes the ovary. These secondary vessels compare with the ovarian spiral arteries originally described in the rabbit.[1,2] They give rise in turn to clusters of still smaller tertiary spiral arteries.

In the course of injecting the blood vessels in a series of about 60 pairs of human ovaries,[5] observations have been made on the distribution and character of the secondary and tertiary blood vessels in the human ovary. It is clear that, in the absence of estrogen these vessels undergo progressive involution and eventually disappear. They are highly developed, dense and numerous only in the presence of estrogen. The evidence for this is as follows:

a) In 4 hypertensive subjects past the menopause, the ovaries were sclerotic, one endometrium atrophic, and there were few or even no secondary and tertiary ovarian spiral arteries.

b) One normotensive subject with a recent menopause had a uterus with an actively proliferating endometrium. Her ovaries were sclerotic, but the secondary and tertiary ovarian arteries were profuse and well developed although the vessels were more widely spaced than in menstruating women.

c) Two hypertensive subjects, not yet past the menopause, possessed non-sclerotic ovaries. The ovarian spiral arteries were luxuriant and profuse.

d) Three normotensive premenopausal women possessed sclerotic ovaries and either proliferative, or early secretory endometria. The secondary and tertiary ovarian spiral arteries were profuse and highly developed.

e) Preparations from 6 fetal and neonatal ovaries showed a moderate to a profuse degree of spiraling of the minute ovarian blood vessels only in full term infants, at a time when, or soon after maternal hormones are known to exert a trophic action on the genital tract of the newborn female.[6]

It is clear from the above, therefore, that only when estrogen is demonstrably present are the ovarian spiral arteries of women well developed. In its absence they are atrophied.

* Aided by a grant from the Kate Lubin Research Foundation, Inc.

† Research Fellow, Department of Obstetrics and Gynecology, Cumberland Hospital.

1 Reynolds, S. R. M., *Am. J. Obst. and Gynecol.*, 1947, **53**, 221.

2 Reynolds, S. R. M., *Endocrinology*, 1947, **40**, 381.

3 Reynolds, S. R. M., *Endocrinology*, 1947, **40**, 388.

4 Delson, B., Lubin, S., and Reynolds, S. R. M., *Endocrinology*, 1948, **42**, 124.

5 Delson, B., Lubin, S., and Reynolds, S. R. M., *Am. J. Obst. and Gynecol.*, in press.

6 Scammon, R. E., Proc. Soc. Exp. Biol. and Med., 1926, **23**, 687.

Hypertensive disease and sclerosis do not, of themselves, give rise to involution of the ovarian spiral arteries.

The significant fact may be mentioned in conclusion that a similar trophic action of estrogen on the endometrial spiraled arterioles is well known from the work of Okkels and Engle[7] (see [8], also). Hence it appears that the action of estrogen upon the vasculature of the genital tract includes not only its generally recognized effects within the uterus,[7] but it includes the ovaries as well. The hormone has, moreover, a particular predilection for spiraled arterial structures in these tissues.

7 Okkels, H., and Engle, E. T., *Acta path. et microbiol. Scandinav.*, 1938, **15**, 150.

8 Reynolds, S. R. M., *J. A. M. A.*, 1947, **135**, 552.

16404

Effect of Vagal Stimulation on Blood Glucose.

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There are conflicting reports in the literature concerning the influence of the vagus nerves on insulin secretion. Some reports[1–4] support the idea that vagal stimulation increases the pancreatic output of insulin. Other work[5–7] fails to substantiate this point of view.

Even with the marked increase in sensitivity to insulin due to hypophysectomy, Keller[8] was unable to produce hypoglycemia in the dog by faradic stimulation of the distal end of the vagi sectioned in the upper abdomen.

These conflicting reports and the studies of Portis and Zitman[9] who attempt to explain the clinical syndromes of weakness and fatigue in psychoneurotic patients on the basis of a vagal induced hyperinsulinism led us to make the following experiments.

Under sodium pentobarbital anesthesia one or both vagus nerves were isolated in the neck. The right vagus was stimulated alone in some dogs; the right and left vagi were stimulated consecutively in some dogs; and the right and left vagi were stimulated simultaneously in some dogs with the tetanizing faradic current. Stimulation was of sufficient strength to cause a marked slowing of cardiac rate as a criterion of vagal effects. Stimulation was continued from 40 minutes to 5¾ hours. Vagal stimulation was continuous except when asystole, cyanosis, apnea, retching or restlessness necessitated either decreasing the current or stopping the stimulation for periods not exceeding 2 minutes in any dog.

Insulin was given subcutaneously to 2 dogs, as indicated in Table I, to be sure that neither the anesthetic nor the vagal stimulation prevented insulin hypoglycemia.

The detailed results are recorded in the table.

Results. When insulin was given subcutaneously to Dog 8 during pentobarbital anesthesia, the usual hypoglycemia resulted. Similarly, when insulin was given subcutaneously to Dog 7 after 3 hours of vagal stimulation, and during continuation of the stimulation, the usual hypoglycemia resulted. This indicates that neither sodium pentobarbital nor vagal stimulation interferes with production of insulin hypoglycemia.

Dog 2 showed a blood glucose of 49 mg % 30 minutes after cessation of vagal stimula-

1 Britton, S. W., *Am. J. Physiol.*, 1925, **74**, 291.

2 Deitrich, S., *Arch. f. exp. Path. u. Pharmakol.*, 1927, **125**, 336.

3 Clark, G. A., *J. Physiol.*, 1925, **59**, 446.

4 LaBarre, J., *Am. J. Physiol.*, 1930, **94**, 13.

5 Phillips, R. A., *Am. J. Physiol.*, 1933, **105**, 257.

6 Long, C. N. H., and Fry, E. G., *Am. J. M. Sc.*, 1933, **185**, 884.

7 Houssay, B. A., Lewis, J. T., and Foglia, V. G., *C. R. Soc. de Biol.*, 1929, **101**, 239.

8 Keller, Allen, personal communication.

9 Portis, S. A., and Zitman, I. H., *J. A. M. A.*, 1943, **121**, 569.

TABLE I.
The Effect of Vagal Stimulation on Blood Glucose in the Normal Anesthetized Dog.

Dog No.		1		1		2		3		4		5		6		7		8		8		9	
Vagal stimulation		(0)		(L then R)		(R)		(R)		(R)		(L and R)		(L and R)		(R)		(0)		(L and R)		(L and R)	
	Time interval after Faradic stimulation	Blood sugar*	Pulse rate	Blood sugar	Pulse rate	Blood sugar	Pulse rate	Blood sugar	Pulse rate	Blood sugar	Pulse rate	Blood sugar	Pulse rate	Blood sugar	Pulse rate	Blood sugar	Pulse rate	Blood sugar	Pulse rate	Blood sugar	Pulse rate	Blood sugar	Pulse rate
Control	—	125	—	82	180	109	204	87	180	76	150	87	180	93	160	75	210	81	—	93	204	112	174
	15	112	—	133	150	100	116	60	96	—	—	—	150‡	—	108	—	82	¶	—	—	84	110	64
	30	125	—	120	168	100	120	113	90§	76	132	97	180	98	120	75	144	—	—	—	—	—	36
	45	112‡	—	89†	180	75	208§	85	180	73	66	—	180	—‡	116	—	70	76	—	93	66	171	96
(1 hr)	60	123	—	121	72§	85‡	—	85	—	78	78	111	168‡	98	110	75‡	90	—	—	—	—	—	—
	75	123	—	92	—	49	—	79‡	—	78	122	—	—	—	104	—	96	48	—	—	—	182	72
	90	126‡	—	120	—	—‡	—	79	—	79	150	122	180‡	94	122	118	125	—	—	100‡	210	—	—
	105	80	—	140	—	79	—	—	—	84	145	—	180	—	108	—	130	—‡	—	—	—	160	84
(2 ")	120	88	—			—	—	—	—	85	72	122	180	—	90	—	110	—	—	—	84	—	—
	135					—	—	—	—	84	108§	—	180	90‡	72	107‡	174	31‡	—	98	84	—	—
	150					—	—	—	—	86	106	120	180	96‡	84	—	108	—	—	101	72§	—	—
	165					—	—	106	—	84	—	—	180	—	—§	—	—	27	—			148	84§
(3 ")	180					85	—	—	—			112	180	99‡	176	104	72	—	—				
	195							—	—			—	—‡	—	—	‖	—	25	—				
	210							—	—			—	—	—	—	94	100						
	225							—	—			127	180§	90	—	64	80						
(4 ")	240							—	—					—	—	—	—						
	255							—	—					—	—	42	174						
	270							—	—					—	—	—	—						
	285							—	—					85	—	36	86§						
(5 ")	300							—	—					—		—	—						
	315							128	—					—		40	198						
	330													—									
	345 (min.)													85									
Duration of exper.				61 (min.)		48		30		166		106		203		184				147		167	
Duration of vagal stimulation				40		48		30		166		66		179		184				127		151	
Period of no stimulation				21		0		0		0		40		24		0				20		16	

* Blood sugar values in mg %. † Left vagal stimulation ended, right vagal stimulation began. ‡Additional doses of 5% sodium pentobarbital, approximately 1 cc amounts. § Vagal stimulation stopped. ‖ 10 U regular insulin given subcutaneously 3½ hr after beginning experiment. ¶ 18 U regular insulin given subcutaneously 15 min. after beginning experiment.

Note: Each dog received 0.5 cc of 5% sodium pentobarbital per kg body wt to produce anesthesia.

Dogs 2, 3, and 4 were fed Purina Chow 5 hr prior to experiment. Dogs 1 and 5 were non-fasting. Dogs 6 to 9 were fasting.

tion. With the exception of this isolated instance in one animal, there is no evidence, from our data on 8 other dogs, that faradic stimulation of either or both vagus nerves produces a hypoglycemia. From this we conclude that it is unlikely that vagal function influences insulin production.

The results were the same whether the animal was fasted 5 hours, 15 hours or fed just prior to the experiment.

16405

A Shaking Device for Multiple Containers.

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The need for simultaneous extraction of a series of fluids in separating funnels occasionally presents itself to the biochemist. In this department, for example, it has been necessary to carry out a number of separations by the Craig[1] counter-current method under conditions in which, either because of volume or tendency to emulsion, the stainless steel device used by Craig was inapplicable.

A piece of equipment has therefore been designed and constructed in the department workshop for the specific purpose of mixing the contents in 250-ml separatory funnels. In view of the comparative simplicity and low material cost of this shaker, as well as the interest it has aroused in workers from other departments, construction details are presented. Obviously, its design may be readily adapted to the use of containers other than separatory funnels.

This shaker (Fig. 1) has been built on a wooden table 24x47 inches, the legs of which are set on casters. Basically, the shaker consists of a frame bolted to a shaft which is turned by a small electric motor. The frame is designed to accommodate 12 separatory funnels in 2 rows, one on each side of the shaft. All structural material is ply-wood except for the half-inch shaft, its bearings, and bearing supports. A simple sheet metal cover over the motor switch has a nail soldered inside to act as a pin which passes through a hole in the shaft. By this device, the frame is held in an upright position when not in use, and accidental throwing of the switch is prevented.

The frame (Fig. 2) consists of 3 ply-wood pieces 9x34 inches. The top piece is removable while the lower 2 are fixed together and fastened to the shaft. The upper and lower pieces are cut from half-inch ply-wood, the center piece from quarter-inch ply-wood.

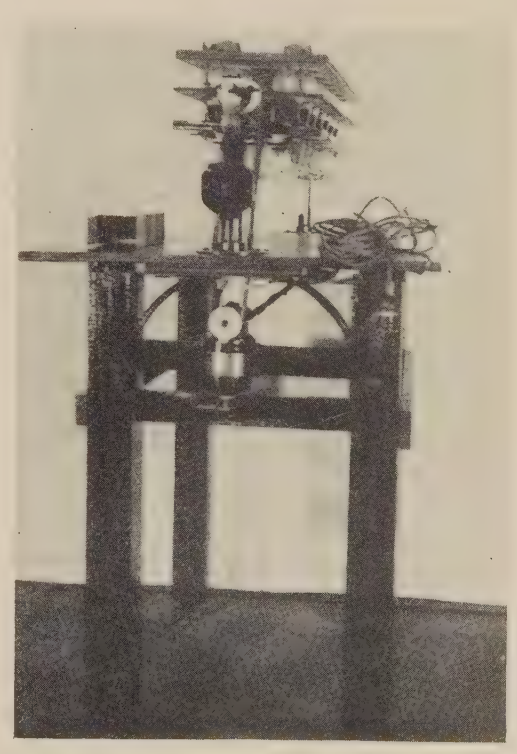

FIG. 1.

1 Graig, L. C., *J. Biol. Chem.*, 1944, **155**, 519.

FIG. 2.

Assembled, the three pieces are spaced 2¼ inches apart. Six quarter-inch bolts 6 inches long are located at the 4 corners and midpoints of the 2 sides to hold the pieces together. Holes are cut in all pieces to serve as mountings for the funnels. The bottom frame piece is cut with key-hole shaped openings, which are lined with sponge rubber, to facilitate removal of the funnels with their stop-cocks. The upper piece is held in place by 6 wing nuts on the quarter-inch bolts. Rubber straps running across the circular holes serve to apply light pressure to the ground glass stoppers as the wing nuts are tightened. Assembled with funnels, the frame is a rigid unit reasonably well balanced about the shaft.

The motor, which is the most expensive part, is mounted below the table top in such a way that the drive pulley is directly below the shaft pulley. A leather V-belt connects the 2 pulleys, passing through a hole in the table top. The 115-volt DC motor (Type RNSH-12 R, Bodine Electric Company, Chicago) is rated at 1/70 horse-power. A gear train incorporated in the motor has a reduction ratio of 25:1, giving an output of 70 rpm.

Some minor design changes are indicated following the construction and use of this shaker. The motor and shaft should be equipped with composite pulleys to permit a selection of shaft speeds. The motor might preferably be turned 90 degrees from its present position so that its armature lies in a horizontal plane. Uniform wear on both armature bearings would result. In bolting the motor to the table, consideration should be given to the necessity of adjusting belt tension.

The total material cost of constructing this shaker, exclusive of the table, was $25 to $30. No special tools were needed that cannot be found in most workshops. Since put into use, the shaker has required a minimum of maintenance.

16406

Penicillin in the Cerebrospinal Fluid Following Parenteral Penicillin.

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(Introduced by L. E. Arnow).

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The desirability of attaining therapeutically significant concentrations of penicillin in the cerebrospinal fluid without the necessity of intrathecal injections is acknowledged. It is probable that some of the complications that have been noted following intrathecal injection of penicillin[1–9] can be assigned to the use of penicillin that was not as highly purified as that currently available and to the use of large doses of penicillin. Nevertheless, it would be advantageous if the procedure of lumbar puncture and the introduction of a foreign substance into the subarachnoid space could be avoided.

It is the consensus that penicillin is transported with difficulty across the tissue barrier between the blood and the cerebrospinal fluid, [10,11,12,14,15] and that inflammation of the meninges may favor passage of penicillin into the cerebrospinal fluid.[16–18] With few exceptions the schedules of penicillin dosage employed in the investigation of the permeability of the meninges with respect to penicillin have not maintained a penicillin plasma concentration high enough to cause diffusion of penicillin into the cerebrospinal fluid. There are reports of moderate doses of penicillin administered either intravenously or intramuscularly, occasionally giving assayable quantities of penicillin in the cerebrospinal fluid,[16–19] whereas, enormous doses of penicillin administered by continuous intravenous drip have produced detectable quantities of penicillin in the cerebrospinal fluid of all patients to whom they have been administered.[20–21] It is suggested, therefore, that penicillin can be recovered in the cerebrospinal fluid of every patient if an amount of penicillin is given to raise the diffusion pressure of penicillin in the plasma above the point at which the hematoencephalic barrier is capable of hindering the passage of the antibiotic into the cerebrospinal fluid. Since a few positive observations have been made following the parenteral adminis-

1 Reuling, J. R., and Cramer, C., *J.A.M.A.*, 1947, **134**, 16.

2 Livingstone, R. G., and Leach, J. E., *Surgery*, 1947, **21**, 683.

3 Walker, A. E., *Arch. Neurol. and Phychiat.*, 1947, **58**, 39.

4 Erickson, T. C., Masten, M. G., and Suckles, H. M., *J.A.M.A.*, 1946, **132**, 561.

5 Siegel, S. T., *J.A.M.A.*, 1945, **129**, 547.

6 Sweet, L. K., *J.A.M.A.*, 1945, **127**, 263.

7 Forrest, A. R., *Brit. Med. J.*, 1945, **2**, 805.

8 Johnson, H. C., and Walker, A. F., *J.A.M.A.*, 1945, **127**, 217.

9 Neymann, C. A., Heckrum, G., and Youmans, G. P., *J. A. M. A.*, 1945, **128**, 433.

10 Fleming, A., *Lancet*, 1943, **2**, 434.

11 Abraham, E. P., Gardner, A. C., Chain, E., Heatley, N. G., Fletcher, C. M., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

12 Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 425.

13 Kaplan, L. I., Read, H. S., Becker, F. T., and Seymour, C. F., *J. Lab. and Clin. Med.*, 1946, **21**, 317.

14 Ory, Edwin, M., Meads, Mawson, Brown, Bruce, Wilcox, Clare, and Finland, Maxwell, *J. Lab. and Clin. Med.*, 1945, **30**, 809.

15 McDermott, Walsh, and Nelson, Russell A., *Am. J. of Syphilis, Gonorrhea, and Venereal Diseases*, 1945, **39**, 403.

16 Kinsman, J. M., and D'Alonzo, C. A., *New Eng. J. Med.*, 1946, **234**, 459.

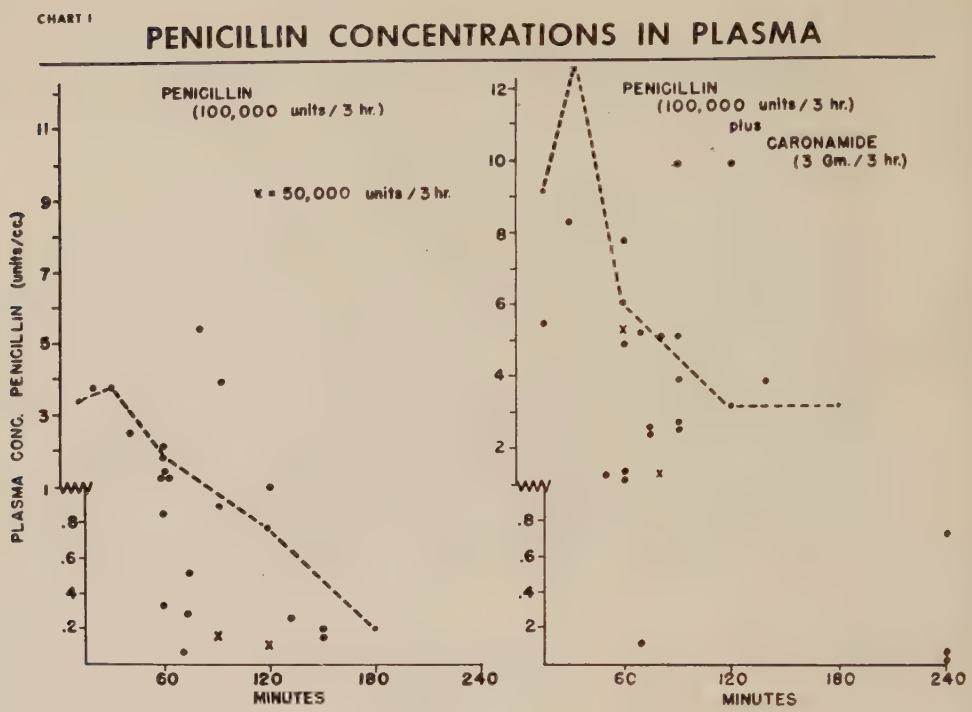
17 Rosenberg, D. H., and Sylvester, J. C., *Science*, 1944, **100**, 132.

18 Cairns, H., Duthie, E. W., Lewin, W. S., and Smith, H. V., *Lancet*, 1944, **1**, 655.

19 Dumoff-Stanley, E., Dowling, H. F., and Sweet, L. K., *J. Clin. Invest.*, 1946, **25**, 87.

20 Schwemlein, G. X., Barton, R. L., Bauer, T. J., Loewe, L., Bundesen, H. N., and Craig, R. M., *J.A.M.A.*, 1946, **130**, 340.

21 Peters, E. E., and Barton, R. L., *Am. J. Syph., Gonorrhea and Venereal Diseases*, 1947, **31**, 522.



tration of 100,000 units of penicillin[16-18] and a group of our patients were receiving this dose every third hour routinely, the following study was made of the penicillin concentrations in the plasma and cerebrospinal fluid.

Patients Studied. Twenty-six patients suffering from central nervous system syphilis (paresis) were treated with 100,000 units of penicillin intramuscularly every third hour for a period of 12½ days. These patients were not selected in any way and they were all in good general health.

Method of Study. One hundred thousand units of penicillin in aqueous solution was administered intramuscularly every third hour for 5 days. Upon the completion of this period of therapy blood and cerebrospinal specimens were obtained simultaneously, the time interval between the last injection of penicillin and the obtaining of samples being accurately noted. Thereafter, the patients continued on the same schedule of penicillin therapy and were given, in addition 3 g of caronamide* orally every 3 hours for an additional 5 days, and then blood and cerebrospinal fluid specimens were again drawn simultaneously. Specimens obtained at the end of the first period were submitted for penicillin assay and those obtained after the second period for both penicillin and caronamide estimations. In 3 patients the period of caronamide therapy preceded that in which penicillin alone was given, and in 2 patients the penicillin dosage administered was 50,000 units every 3 hours rather than 100,000 units. Penicillin in the plasma and cerebrospinal fluid was estimated by a modification of the Rammelkamp serial dilution method making use of a standardized strain of a Group A hemolytic streptococcus as the test organism. Caronamide estimations were done by the Ziegler method.[22]

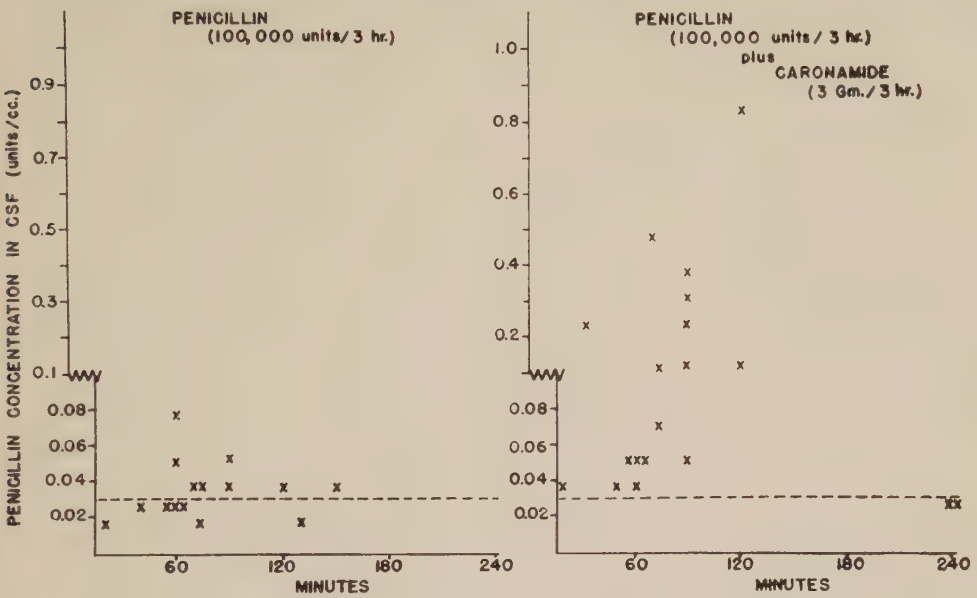
Results. The results obtained from this study are presented in Charts 1 and 2, and Table I. Following the 5 days of penicillin therapy alone, 15 of 23 patients showed assayable quantities of penicillin in the cerebrospinal fluid. The range of concentrations

* Caronamide (4'-carboxyphenylmethanesulfonanilide) used in this investigation was supplied through the courtesy of Sharp & Dohme, Inc.

22 Ziegler, C., and Sprague, J. M., *J. Lab. and Clin. Med.*, 1948, **33**, 96.

CHART II

PENICILLIN CONCENTRATIONS IN CEREBROSPINAL FLUID



found was from 0.019 to 0.052 units of penicillin per cc. Following a period of 5 days therapy with the same doses of intramuscular penicillin and 24 g of oral caronamide per day, 20 of 25 patients showed detectable quantities of penicillin in the cerebrospinal fluid. The range of concentrations was from 0.026 to 2.5 units per cc. Comparison of penicillin concentrations in plasma and cerebrospinal fluid in the two periods of study indicates the enhancement effect of caronamide (Charts 1 and 2).

The estimations of caronamide in the plasma showed that concentrations ranging from 1.2 to 110 mg per 100 cc were obtained in this group of patients. Despite the broad range of caronamide concentrations in the plasma, the compound was detected in the cerebrospinal fluid in significant quantities in only 2 cases (Table I).

Discussion. Inasmuch as the largest group of patients heretofore reported in whom detectable amounts of penicillin were found in the cerebrospinal fluid received from 10 to 25 million units of penicillin by continuous intravenous drip over a period of 24 hours,[20] and lesser doses, except in occasional cases, had failed to show penetration of penicillin into the cerebrospinal fluid, the results of this study are of interest. The individuals studied were regarded as having no inflammation of the meninges, for in no case was the cell count in the cerebrospinal fluid higher than 25 cells per cc. There are precedents for considering patients suffering from general paresis as having a "normal" blood brain barrier,[13,16,17] and it has been shown that the meninges of such "normal" patients are less permeable to the passage of fluorescein than patients suffering from meningitis.[23]

The concentrations of penicillin that were obtained in the cerebrospinal fluid in this study may be regarded as therapeutically significant inasmuch as 0.03 units per cc "is adequate to sterilize actively growing cultures of almost all strains of gonococcus, Group A hemolytic streptococcus, and pneumococcus and most strains of alpha hemolytic streptococcus, about half of the strains of meningococcus, and a somewhat smaller proportion of strains of pathogenic staphylococci."[14] Penicillin concentrations in the cerebrospinal fluid

[23] Lange, K., Schwimmer, D., Boyd, L. J., *Am. J. Med. Sci.*, 1946, **211**, 611.

TABLE I

Concentrations of Caronamide in Plasma and Cerebrospinal Fluid (CSF)

	Time (Min.) after last dose of caronamide							
	30	60	70	75	80	90	120	240
Plasma Concentrations of Caronamide mg./100 cc.	71	110[a]	59[c]	10[d]	21	41[c]	57[c]	1.2[d]
	59	48		77[c]		48	34	
		36					82	
		51[c]						
		58						
		87[b]						
		9[d]						
		57						
		63[c]						
AVERAGES	65	57	59	43	21	44	57	1.2

a—Caronamide concentration in CSF was 48 mg./100 cc

b—Caronamide concentration in CSF was 4.5 mg./100 cc.

c—Caronamide concentration in CSF was "a trace" (less than 1 mg./100 cc.)

d—These caronamide concentrations are far below those obtained in the other patients in this study and cannot be correlated with previous experience. There is some likelihood that patients did not receive scheduled medication

indicated in Table I as <0.026 and <0.039 have been regarded as zero values in determining the number of patients showing a significant amount of antibiotic in the cerebrospinal fluid. Actually, these patients may have had quantities of penicillin in the cerebrospinal fluid that were undetectable by the method of penicillin assay employed. Since the patients studied in this investigation had been and were being treated with malaria and the Kettering hypertherm, it was of importance to determine whether these circumstances favored the retention of penicillin in the circulation. In Chart 1 the plasma concentrations of penicillin obtained in this group of patients have been plotted against values that were obtained in a group of normal individuals following the administration of 125,000 units of penicillin in a control and a caronamide modified period. The fact that the plasma concentrations obtained in this group of patients fell, in the main, below those obtained on normal subjects (left side, Chart 1) clearly indicates that the group did not deviate markedly from previously studied normal groups of patients with respect to the rapidity with which penicillin was excreted. The higher plasma concentrations obtained during the periods of caronamide administration compare favorably with the enhancement effect of caronamide (right side, Chart 1) observed in previously studied normal patients. The increase of penicillin plasma concentrations by 2- to 7-fold[24,25] by the co-administration of caronamide and penicillin was confirmed.

In Chart 2 the concentrations of penicillin observed in the cerebrospinal fluid in the control and in the caronamide modified periods have been plotted and the tendency to higher values following the administration of caronamide is clearly indicated. It is noteworthy that the concentrations of penicillin in the cerebrospinal fluid observed in this study following the administration of 800,000 units of penicillin per day, are of the same

[24] Crosson, J. William, Boger, William P., Shaw, Christopher, C., and Miller, A. Kathrine, *J.A.M.A.*, 1947, **134**, 1528.

[25] Boger, William P., and Baker, Richard M., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 1.

magnitude as those obtained following the administration of 20 to 25 million units by continuous intravenous drip over a 24-hour period.[20] In this preliminary investigation repeated sampling of the plasma and the cerebrospinal fluid following the administration of a single penicillin dose in order to determine the measure to which plasma and cerebrospinal fluid penicillin concentrations can be correlated, was not done, and it should be emphasized that the cerebrospinal fluid concentrations (Chart 2) are not a direct reflection of the concentration of penicillin found in the plasma at the time that the specimens were simultaneously obtained in this study. Since penicillin probably gains access to the cerebrospinal fluid through the choroid plexuses and a measurable time is required for penicillin in the ventricular cerebrospinal fluid to diffuse into the lumbar cerebrospinal fluid,[19] the distribution of penicillin between plasma and cerebrospinal fluid was probably determined by much higher levels of penicillin in the plasma than those observed at the times when specimens were obtained. Repeated samplings of blood and cerebrospinal fluid following a given dose of intramuscular penicillin are being done at the present time in an effort to determine a more exact plasma-cerebrospinal fluid penicillin concentration relationship, and the results will be the subject of a subsequent report.

Plasma penicillin concentrations above 0.156 units for as long as 4 hours with a peak concentration during that 4 hour period up to 10 to 20 units of penicillin per cc have resulted in a concentration of at least 0.02 units per cc in the cerebrospinal fluid.[15] These results were observed in 5 patients, all of whom received at least 300,000 units of penicillin intramuscularly and 100,000 units intravenously. These peak concentrations correspond closely to the concentrations that were maintained for 24 hours by the continuous intravenous administration of from 10 to 25 million units of penicillin, and that resulted in measurable quantities of penicillin in the cerebrospinal fluid.[20,21] Lesser doses of penicillin, 15,000 to 20,000 units, either intramuscularly or intravenously,[12] 30,000 units intramuscularly[13] and 20,000 to 60,000 units intramuscularly[14] cannot be expected to give such peak concentrations for even a short period of time and these levels of dosage have not given assayable quantities of penicillin in the cerebrospinal fluid. One group of investigators[17] has indicated rather high levels in the cerebrospinal fluid following intravenous doses of 20,000 to 40,000 units, but some question has been raised about the reliability of the Foster turbidometric method of penicillin assay. A single injection of 100,000 units intravenously in a case of meningitis resulted in detectable quantities of penicillin in the cerebrospinal fluid within 2 hours,[18] and such an injection can be assumed to have given a concentration of penicillin in the plasma above 10 units per cc for only a few minutes, and above 5 units for approximately 30 minutes.[26] Five hundred thousand units given intravenously resulted in 0.039 units per cc in the cerebrospinal fluid at the end of 2 hours and such a dose resulted in plasma concentrations from 87 to 10 units over a period of 30 minutes and above 1.5 units throughout the 2-hour period prior to the sampling of the cerebrospinal fluid.[26]

The tissue barriers between the cerebrospinal fluid and blood prevent the free exchange of penicillin between the plasma and the cerebrospinal fluid, but they are not impermeable to penicillin. It can be anticipated that there will be individual differences both in the concentration of penicillin that must be achieved in the plasma and the duration of time over which this concentration must obtain, to cause diffusion of penicillin into the cerebrospinal fluid, but it should be possible to obtain with regularity therapeutically significant concentrations of penicillin in the cerebrospinal fluid following parenterally administered penicillin. From data reported in the literature and from the results of this study, there is reason to believe that a plasma concentration of 10 units per cc maintained for less than 4 and possibly less than 2 hours is sufficient to cause measurable amounts of antibiotic to appear in the cerebrospinal fluid. The rapidity with which penicillin can be made to appear in the cerebrospinal fluid is

[26] Boger, William P., Unpublished data.

of great importance in the therapy of meningitis, and it is suggested that intermittent intravenous injection of penicillin may most promptly establish the circumstances favoring diffusion of penicillin into the cerebrospinal fluid.

In this investigation the use of oral caronamide to produce an inhibition of excretion of penicillin by the renal tubules resulted in increased penicillin plasma concentrations and, in the measure that it did so, contributed to the finding of larger quantities of penicillin in the cerebrospinal fluid. The failure of caronamide to pass freely into the cerebrospinal fluid in the majority of patients is another evidence that there is a hindrance of the free interchange of dissolved substances between the blood and the cerebrospinal fluid. The passage of caronamide into the cerebrospinal fluid when excessively high concentrations of caronamide were obtained in the plasma suggests that the barrier is only relatively impermeable.

Although the plasma concentrations of caronamide observed in this study were in excess of the 20 to 40 mg per 100 cc that have been shown to be effective in inhibiting the excretion of penicillin,[27] the only toxic symptoms were nausea and vomiting in 5 patients, and in only 2 patients was it necessary to discontinue administration of the drug.

Conclusions. Therapeutically significant quantities of penicillin (above 0.03 units per cc) have been observed in the cerebrospinal fluid following the intermittent intramuscular injection of 100,000 units of penicillin in aqueous solution. Caronamide increased the plasma levels of penicillin resulting from the administration of this dose of penicillin and thereby increased the amounts of penicillin found in the cerebrospinal fluid. Caronamide itself enters the cerebrospinal fluid only at excessively high plasma concentrations (87 to 110 mg per 100 cc).

The authors wish to express their indebtedness to the Chiefs of the Neurological Services at the Philadelphia General Hospital for their kindness in granting permission to study patients on their services, to Grace Schuchardt for caronamide determinations, and to Elizabeth Hughes and June E. Heckman for penicillin assays.

[27] Boger, William P., Trans. and Studies College of Physicians of Phila., 1947, **15**, 104.

16407

Influence of Dietary Lipids on Experimental Tuberculosis.*

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Experiments are recorded in the literature in which it has been demonstrated that the administration of certain of the lipids may result in an enhancement of experimental tuberculosis while the administration of other of these compounds may result in a retardation of the progress of the infection.

Weigert[1] conducted feeding experiments, using guinea pigs, and concluded that the tuberculous processes generalized more rapidly in animals fed a strict carbohydrate diet than in those receiving an added liberal amount of milk fat.

Troteanu[2] administered cod-liver oil directly into the stomach of guinea pigs and noted an enhancement of the progress of tuberculosis following the inoculation of the animals intraperitoneally with 1 mg of bovine

* This paper represents a portion of the dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, St. Louis University.

[1] Weigert, R., *Berl. Klin. Wechen.*, 1907, **44**, 1209.

[2] Troteanu, V. C., *Comp. Rend. Biol.*, 1929, **102**, 141.

TABLE I.
Saponification and Iodine Values of Body Fat of Groups of Albino Mice Which Had Been Held on Their Respective Rations for a Period of 10 days.

Diet	Saponification value		Iodine value	
Non-lipid ration	202.2, 202.3		78.4,	78.3
Coconut oil "	+15.7%*	234.0, 234.0	—12.6%*	68.4, 68.4
Olive " "	0.0%*	202.0, 202.0	+ 7.9%*	84.6, 84.6
Oleic acid "	—1.0%*	201.8, 201.9	+33.9%*	105.0, 105.0
Linseed oil "	0.0%*	202.5, 202.5	+33.9%*	105.0, 105.0
Oils:				
Coconut oil	253.0, 253.0		9.7,	9.7
Olive oil	193.5, 193.5		83.7,	83.7
Oleic acid	195.5, 195.6		91.4,	91.4
Linseed oil	195.0, 195.0		180.0,	180.0

* The percentage increase or decrease in saponification and iodine values of the body fat of animals receiving the coconut oil fatty acids, olive oil, and linseed oil rations as compared with values of the body fat of the animals fed the non-lipid ration.

tubercle bacilli.

Negre[3] reported that the ingestion or subcutaneous injection of cod-liver or olive oil resulted in an enhancement of experimental tuberculosis in guinea pigs and rabbits.

Negre, Berthelot and Bretey[4] injected the ethyl esters of a series of fatty acids subcutaneously into tuberculous guinea pigs. The ethyl esters of palmitic, myristic, lauric, arachidic, capric, and stearic acids retarded the appearance of tuberculous lesions.

The present experiment consists of an investigation of the progress of experimental tuberculosis in groups of mice whose body fat had been changed by the administration of a basal ration containing various types of lipids.

Methods. Male, Swiss albino mice 90 days of age were used in this experiment. The lipids were administered to these animals in the form of oleic acid,† olive oil,‡ linseed oil,§ and the total fatty acid of coconut oil.† Each of the lipids was mixed with a fat-free, casein-supplemented ration[5] in a concentration of 20%. The 4 lipid-containing rations and the non-lipid ration were administered to groups of 25 mice. After having been fed their respective rations for a period of 10 days, 5 animals were removed from each of the 5 groups. These animals served as dietary controls. A second group of 5 animals was selected from each of the groups. The body fat of these animals was utilized for a determination of the saponification and iodine values which are recorded in Table I. The remaining 15 animals in each of the 5 groups were inoculated intravenously with .5 mg of MS-45 strain‖ of *Mycobacterium tuberculosis.* A large inoculum was used since these animals were known to be resistant to experimental tuberculosis.

Average weights of each of the groups of animals were recorded at the time of inoculation. Recordings were also made of the individual weight of each animal at death.

The animals remaining alive at the end of 37 days were sacrificed by exposure to ether vapor. Sections were prepared from the lungs, liver, spleen and kidney tissue of each animal succumbing during the experimental period and from the tissues of those which were sacrificed. Each section was stained with hematoxylin and eosin. Certain of the

[3] Negre, L., *Annal. de l'Inst. Pasteur,* 1932, **49**, 319.

[4] Negre, L., Berthelot, A., and Bretey, L., *Ann. Inst. Pasteur,* 1937, **59**, 457.

† 0-18 "Elaine" and F-716 Coconut Fatty Acids generously supplied by Emery Industries, Inc., Cincinnati, 2, O.

‡ "Torino Brand," J. Ossola Co., New York City.

§ Raw Linseed Oil, U. S. Paint, Lacquer and Chemical Co., St. Louis, Mo.

[5] Pearce, E. L., *et al., Jour. Biol. Chem.,* 1947, **168**, 271.

‖ This strain of *Mycobacterium tuberculosis* was isolated in 1945, from a tuberculous guinea pig which had been inoculated with a sputum specimen originally obtained from Mount St. Rose Sanatorium, St. Louis, Mo.

TABLE II.
Average Weights, Survival, and Average Degree of Pathological Involvement of Tissues of Albino Mice Infected with 0.5 mg of *Mycobacterium tuberculosis*.

	Mice	No. surviving after 37 days	Wt when infected (g)	Wt at death (g)	% of substance occupied by lesions*			
					Lungs	Liver	Spleen	Kidney
Non-lipid ration	15	15†	21.5	19.4	1X	—X	—X	—X
Coconut oil "	15	13†	20.5	17.1	1.3X	1X	—X	—X
Olive " "	15	4†	20.4	13.5	2X	1.3X	2.1X	—X
Oleic acid "	15	5†	20.0	14.1	2.4X	1.3X	1.5X	—X
Linseed oil "	15	4†	19.7	12.3	2.8X	1.5X	—X	—X

* "—X" indicates that less than 25% tissue involvement, "1X" denotes 25%, "2X" 50%, "3X" 75%, and "4X" pathological involvement exceeding 95%.
† Animals sacrificed at the end of 37 days.

sections were also stained by the Ziehl-Neelsen carbol fuchsin technic for acid-fast organisms. The extent of the tuberculous processes in each of the tissues was graded on the following basis: "O," apparently normal; "-X," less than 25% involved pathologically; "1X," 25% involved; "2X," 50% involved; "3X," 75% involved; "4X," exceeding 95% pathological involvement.

The saponification and iodine values indicated that a significant change had occurred in the body fat of the albino mice. In a comparison with the non-lipid ration, the administration of the coconut oil fatty acid ration resulted in a decrease in the iodine value and an increase in the saponification value indicating a deposition of short-chain, saturated acids in the body fat. There was no increase in the saponification values in the case of the olive oil, oleic acid, and linseed oil rations, however, the iodine value increased indicating a deposition of unsaturated acids. The iodine value for the body fat of animals fed the oleic acid ration is inconsistent with the corresponding value for oleic acid. This apparent discrepancy may have been due to the presence in the oleic acid of a saturated and highly unsaturated acid, *e.g.*, stearic and linolenic acid, in which the unsaturated acid was preferentially absorbed from the intestinal tract of the animal.

The histological picture differs from that described by Youmans and McCarter[6] in the extent of the coagulative necrosis occurring in the liver tissue and in the occurrence of tubercles in the medulla of the kidney.

Sections stained by the Ziehl-Neelsen technic revealed the presence of acid-fast bacilli occurring singly or in clumps in all lesions.

The albino mice proved to be resistant to experimental tuberculosis when fed the non-lipid ration. Of the 5 groups of animals, those receiving the non-lipid ration exhibited the maximal survival, a minimal loss of weight, and a minimum of pathological involvement in the lungs, liver, spleen and kidney tissues. Previous experiments indicate that the administration of a non-lipid ration to mice, which are known to be susceptible to experimental tuberculosis, does not change the state of susceptibility of such animals. The observed susceptibility of one strain and the resistance of a second strain of mice when subjected to identical non-lipid dietary regimens, may represent an expression of variation in the native resistance of the species.

The results obtained following the administration of the ration containing the total fatty acids of coconut oil were similar to those which resulted from administering the non-lipid ration. However, there was a slight increase in weight loss and mortality. There was also a slight increase in the pathological involvement of the lungs and liver. The pathological involvement of the spleen and kidney tissues was not different from that of the animals receiving the non-fat ration.

As compared with the non-lipid and coconut fatty acid rations, the administration of the olive oil, oleic acid, and linseed oil rations resulted in a significantly greater mortality,

[6] Youmans, G. P., and McCarter, J., *Amer. Rev. Tuberc.*, 1946, **52**, 432.

TABLE III.
Wt of the Control Series of Albino Mice.

Diet	Avg wt at time of inoculation of test mice	Avg wt after 37 days	Increase in wt (%)
Non-lipid ration	21.4	24.5	12.6
Coconut oil "	18.2	18.5	1.6
Olive " "	19.6	22.0	11.8
Oleic acid "	22.0	24.4	10.9
Linseed oil "	24.2	25.0	3.3

weight loss, and pathological involvement of the lungs, liver, spleen and kidneys. The most extensive lung involvement was observed in those animals receiving the linseed oil ration. The liver and kidney tissue of those animals receiving the olive oil, oleic acid and linseed oil rations displayed an equally severe degree of pathological involvement. The extensive involvement of the spleen tissue of those animals receiving the olive oil and oleic acid rations was striking as compared with the involvement observed in the spleen tissue of the remaining 3 groups of animals.

Approximately equal increases in weight occurred in those animals receiving the non-lipid, the olive oil, and the oleic acid rations. There was little change in the weight of the mice receiving the linseed oil and coconut oil fatty acid rations.

Discussion. It was the purpose of this experiment to effect a deposition of fatty acids in the body fat of the mice corresponding to those of the administered diet. Under such conditions any enhancement or retardation of experimental tuberculosis in the animal could be ascribed to the type of fatty acids present in the body fat. In the case of the non-lipid ration, the fatty acids present in the body fat would be limited to those synthesized from the carbohydrate fraction of the ration.

In a study of the growth of *Mycobacterium tuberculosis* in trypticase[7] and in trypticase-albumin media[8] containing a series of fatty acids,¶ it was found that capric, lauric, palmitic, and myristic acids were inhibitory at concentrations of 10^{-5}% in the former media and at concentrations of 10^{-3}% in the latter media. Growth of *Mycobacterium tuberculosis* occurred at 1% or at 0.1% concentrations of each of the remaining fatty acids of the group tested.

The results of the *in vitro* investigation correlate with the results of this *in vivo* experiment. The administration of the coconut oil fatty acid ration to mice resulted in an enhanced resistance to experimental tuberculosis as compared with the administration of olive oil, oleic acid and linseed oil ration to these animals. Chemical analyses have shown that coconut oil contains approximately 7% capric, 46% lauric, 19% myristic, and 10% palmitic acids. The presence of significant amounts of caprylic, stearic and oleic acids in this oil may be responsible, in part, for the few fatal cases observed in the animals receiving the coconut oil ration.

It has been noted that growth of MS-45 strain of *Mycobacterium tuberculosis* occurs *in vitro* in the presence of 1% concentrations of oleic and linoleic acids. This correlates with the fact that the administration of olive oil and linseed oil rations to mice resulted in increased susceptibility to experimental tuberculosis. The results of the administration of the oleic acid ration was not significantly different from the results obtained by administering the olive oil ration which indicated that oleic acid was the factor responsible for enhanced susceptibility to experimental tuberculosis.

In view of the high percentage of long chain, unsaturated fatty acids composing cod-liver oil, the question arises as to the effect of this

7 Dubos, R. J., and Davis, B. D., *Jour. Exp. Med.*, 1946, **83**, 409.

8 *Ibid.*

¶ Formic, acetic, propionic, n-butyric, iso-valeric, iso-caproic, n-heptylic, caprylic, pelargonic, capric, lauric, myristic, palmitic, stearic, oleic, and linoleic acids.

oil, *per se*, on the progress of tuberculous infection.

Schoenheimer[9] has shown that the fatty acids of the diet are first incorporated into body fat before they are oxidized. Thus, the body fat is part of a dynamic system, the fatty acids being continually deposited and withdrawn.

Franke, *et al.*,[10] found that of all the substrates tested manometrically, certain of the fatty acids effected the greatest increase in respiration of *Mycobacterium tuberculosis* despite the fact that other of these acids were inhibitory under such conditions.

Due to the fact that *Mycobacterium tuberculosis* is located extra-cellularly in its host, this microorganism has access to the body fluids of the host. Since the body fat is in a dynamic state, such fluids will contain fatty acids corresponding to those of the diet. If the diet contains large amounts of fatty acids which serve to increase the respiration of *Mycobacterium tuberculosis*, enhanced proliferation of the organism would be expected to occur. However, if the diet contains large amounts of fatty acids which serve to inhibit the respiration of *Mycobacterium tuberculosis*, a retarded proliferation of the organism would be expected to result.

Thus, the type of fatty acids present in the diet may determine whether or not conditions are favorable for the development of tuberculous infection.

Summary. 1. The administration of a non-lipid, casein-supplemented ration to resistant Swiss albino mice retarded the progress of experimental tuberculosis. 2. Experimental tuberculosis was also retarded by the administration of the ration to which 20% of the total fatty acids of coconut oil had been added. 3. Olive oil, linseed oil, and oleic acid enhanced the progress of experimental tuberculosis when these compounds were added to the ration in a concentration of 20%.

9 Schoenheimer, R., and Rittenberg, D., *Jour. Biol. Chem.*, 1936, **114**, 381.

10 Franke, W., and Schillinger, A., *Biochem. Z.*, 1944, **316**, 313.

16408

A Lipid Anticoagulant from Brain Tissue. Physiochemical Characteristics and Action *in vitro* and *in vivo*.*

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Dilution of a crude aqueous brain extract or of a suspension of its lipid (cephalin) fractions often improve their clot accelerating power. This apparent paradoxical behavior of strong thromboplastic solutions has been frequently observed.[1,2,3] Attempts to separate the clot inhibiting from the clot accelerating substances have only been moderately successful.[3,4] The experiments here reported indicate that it is possible to separate from brain tissue, by a suitable method of extraction, a heat labile inhibitor of blood coagulation, probably of a lipid nature and acting *in vitro* and *in vivo* as a powerful antithromboplastin.

Reagents. Thromboplastin. 300 mg of acetone dried human brain powder are extracted for ½ hour with 5 cc of 0.85% sodium chloride at 50°C. The supernatant fluid is centrifuged at 1000 R.P.M. for 2 minutes and tested against recalcified citrated plasma.

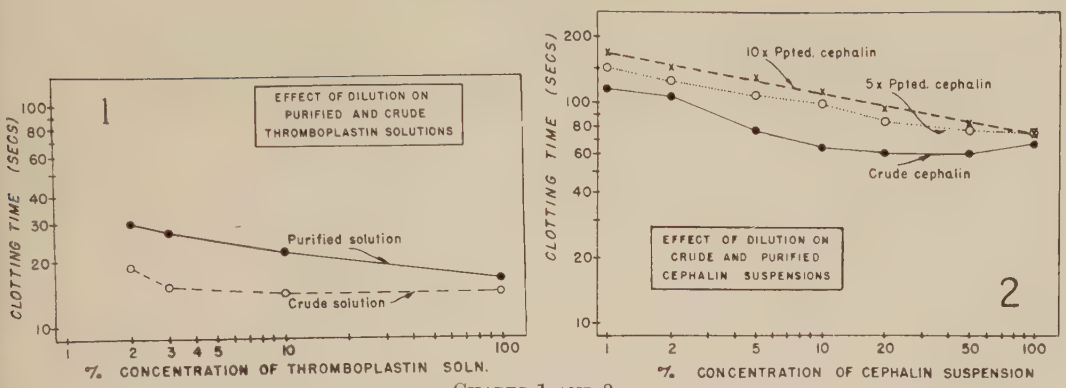
* Aided by grant from the U. S. Public Health Service.

1 Aggeler, P. M., and Lucia, S. F., *Am. J. Med. Sci.*, 1940, **199**, 181.

2 Kazal, L. A., and Arnow, L. E., *Arch. Biochem.*, 1944, **4**, 183.

3 Chargaff, E., *J. Biol. Chem.*, 1937, **121**, 175.

4 De Suto Nagy, G. J., *J. Biol. Chem.*, 1944, **156**, 433.



CHARTS 1 AND 2.

The clot accelerating action of intact and diluted thromboplastin and cephalin extracts. 0.1 cc of thromboplastin (or cephalin), 0.1 cc plasma, 0.1 cc 0.02 M $CaCl_2$.

Thromboplastin solutions were purified by repeated precipitations of the thromboplastic lipoprotein by dilution and acidification.[5] Cephalin suspensions were prepared by a method previously described; cephalin was purified by repeated precipitations of the lipid in its ether solution with cold absolute ethanol.[6] Unless otherwise stated, all tests were carried out in collodion coated tubes, 13 mm i.d. at 38°C. Citrated (0.38%) human plasma was collected with the precautions outlined elsewhere[7] and kept in paraffin tubes until tested.

1. *Activity of crude and purified aqueous thromboplastin solutions and cephalin suspensions after dilution.* As shown in Charts 1 and 2, crude preparations of thromboplastin and cephalin gain in clot accelerating potency on dilution, until a point is reached beyond which further dilution results in loss of clot promoting power. Purified preparations, however, though originally less potent than the crude ones, do not gain clot accelerating power upon dilution. It would seem, therefore, that during the course of purification a clot inhibitor is removed.

2. *Separation of the Anticoagulant Fraction from Brain Tissue.* The methods employed take advantage of the solubility characteristics of the clotting inhibitor and avoid any procedure which may involve raising the temperature beyond 50°C, since it was found early in the work that the inhibitor is heat labile.

First Method. 30 g of acetone dried human brain powder capable of passing through a 20-mesh sieve are extracted with 450 ml of absolute ether, in the cold, for 7 days with occasional shaking. The supernatant is filtered and concentrated *in vacuo* to a volume of 50-75 ml. To this sample is added sufficient cold absolute ethanol for maximum precipitation (approx. 125-150 ml). After standing in the refrigerator for 30 minutes it is centrifuged at 1500 R.P.M. for 5 minutes. The supernatant is decanted off and saved. The residue is dissolved in 20 ml of absolute ethyl ether. The precipitation with cold absolute ethanol is repeated, this time adding 110-125 ml of the alcohol. This is handled as before, again saving the supernatant and dissolving the residue in 20 ml of ether. A third precipitation is done, the combined supernatants are filtered and the filtrate distilled *in vacuo* in a flask immersed in a water bath maintained at 46-48°C. The residue remaining in the flask after the distillation is completed is removed with absolute ethyl ether. The ether is evaporated off, 50 ml of acetone added to the residue and the mixture is allowed to remain in contact at 5°C overnight. The acetone is decanted off and the acetone insoluble residue is dried *in vacuo*. A golden yellow powder results to which is

[5] Seegers, W. H., Brinkhous, K. M., Smith, H. P., and Warner, E. D., *J. Biol. Chem.*, 1938, **126**, 91.

[6] Tocantins, L. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 44.

[7] Tocantins, L. M., *Am. J. Physiol.*, 1943, **139**, 265.

TABLE I.
Relative Anticoagulating Potency of Lipid Inhibitor Fractions Obtained by Different Methods.

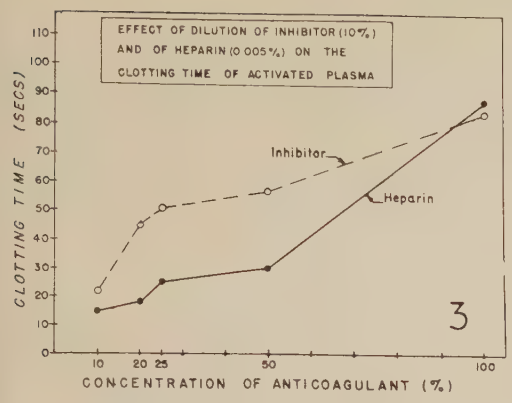
	Lipid inhibitor fractions				
	1st method	2nd method			Control
	Lot A	Lot B	Lot C		NaCl
Strength of solution, g %	50	10	10	50	0.85
Duration of extraction (days)	7	1	5-6		
Temp. of extraction, °C	5	20-22	5		
Heparin units/mg	0.25	0.5	0.75		
Clotting time:					
(a) Plasma + inhibitor sol. + $CaCl_2$	>24 hr	>24 hr	>24 hr	>24 hr	—
(b) Plasma + thrombopl. + inhib. sol. + $CaCl_2$	160 sec.	85 sec.	380 sec.	>24 hr	
(c) Plasma + $CaCl_2$ + 0.85% NaCl	—	—	—	—	500-610 sec.
(d) Plasma + thrombopl. + $CaCl_2$ + 0.85% NaCl	—	—	—	—	14 sec.

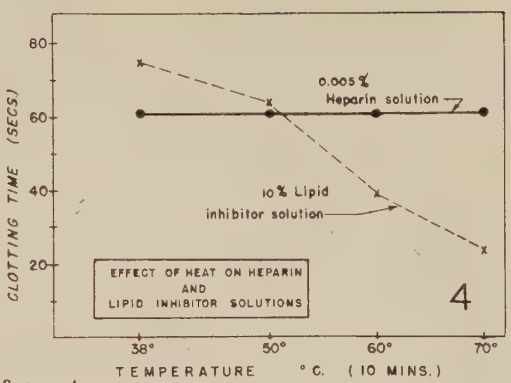
added 30 ml 0.85% NaCl. This is macerated with a mortar and pestle to make the solution as uniform as possible and then homogenized by putting through a colloidal mill 7-10 times. The pH of the solution is adjusted to 7.2-7.4 and it is then ready for use. The acetone soluble portion, on evaporation, yields a white powdery substance which has clot accelerating activity.

Second Method. 30 g of acetone dried brain powder are extracted for 5-6 days at 5°C with 600 ml of absolute methanol. The supernatant is filtered and the filtrate distilled *in vacuo* in a flask immersed in a water bath at 38-40°C. The residue remaining in the flask after the distillation is completed is removed with absolute ethyl ether. The ether solution is kept at 5°C overnight during which a white precipitate settles out. The supernatant is decanted off and the precipitate washed with cold ether. The combined ether extracts are evaporated off *in vacuo* leaving a creamy white, waxy powder. A 10 g% solution is made in 0.85% NaCl, put through a homogenizer 7-10 times and the pH adjusted to 7.2-7.4. The potency of the inhibitor seems to be related to the proportion of clot accelerating substances present and the latter seems to go in solution better at temperatures higher than 5°C.

The results of testing the activity of 3 lots obtained by the 2 methods above described are shown in Table I. The anticoagulating potency of the inhibitor has been compared with that of heparin (Connaught Laboratories, Toronto) 1 mg of which contains 100 units. Though the mechanism of action of the lipid inhibitor differs from that of heparin, comparison with it provides a general measure of its anticoagulant potency, in terms of that of a well known substance. Our most potent products have 0.75 heparin units per mg (Table I) or an activity 133 times less than that of crystalline heparin. Comparison of the 2 anticoagulants was done by noting their relative potency in delaying coagulation of recalcified citrated plasma activated by a strong thromboplastin solution. If 10 mg of the lipid inhibitor prolongs the clotting time of activated plasma from 14 to 80 seconds and this prolongation corresponds to that brought about by 5 units (or 0.05 mg) of heparin, then 1 mg of the lipid inhibitor may be considered to have ½ heparin unit. That this is only a rough method of comparison is shown in Chart 3. Dilution of the 2 anticoagulants shows that in the lower concentrations, the lipid inhibitor is a more effective antithromboplastin than the heparin.

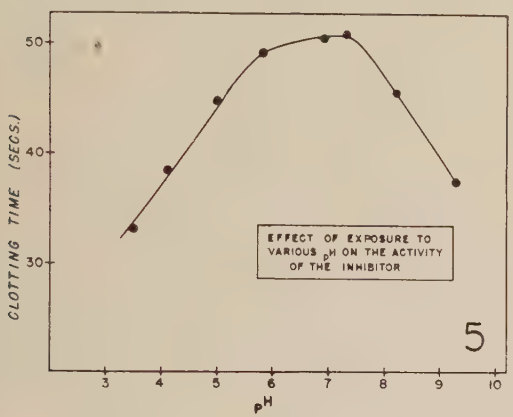
3. *Effect of Heat and pH Changes.* Unlike heparin, the lipid inhibitor is heat labile. Temperatures of 65-70°C for 10 minutes almost entirely destroy the activity of the inhibitor in 0.85% NaCl (Chart 4). Exposure





CHARTS 3 AND 4.

Comparison of the effect of dilution or heat on the action of heparin and the lipid inhibitor. 0.1 cc thromboplastin, 0.1 cc heparin (or inhibitor), 0.1 cc plasma, 0.1 cc 0.02 M $CaCl_2$.



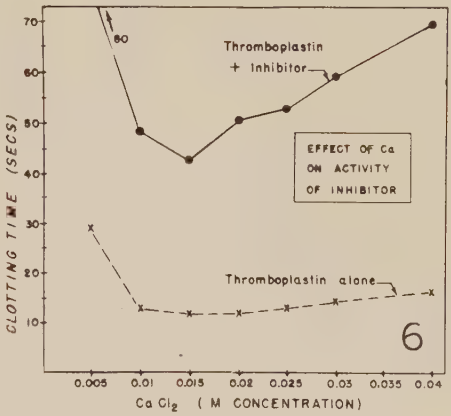


CHART 5.

The pH of a 10% solution of the inhibitor was kept at various levels for 180 min. by addition of dilute HCl or NaOH. It was then brought back to 7.2 and tested.

CHART 6.

0.1 cc thromboplastin, 0.1 cc 10% lipid inhibitor (or 0.85% NaCl), 0.1 cc plasma, 0.1 cc $CaCl_2$ (various M concentrations). The optimum range of Ca concentration is narrower for the clotting system containing the inhibitor, but in other respects does not differ from the system without the inhibitor.

to an excessively acid or alkaline medium likewise reduces the anticoagulating potency of the lipid inhibitor (Chart 5).

4. *Mode of Action.* The lipid extract seems to owe its clot delaying action to a reduction of the activating effect of thromboplastin on prothrombin (Table II). In contrast with heparin, it has no effect on thrombin even when a 20% solution of the lipid inhibitor is used (Table II). It does not seem to bind calcium (Chart 6) and does not alter the rate of conversion of fibrinogen to fibrin by thrombin. Since an antiprothrombic effect is difficult to distinguish from an antithromboplastic one, it is not possible to be certain in this regard. The fact that it does not block the activation of prothrombin by Russell viper venom may indicate that it does not have an antiprothrombin effect. The natural plasma antithromboplastin is likewise impotent against the clot accelerating action of Russell viper venom.[8]

5. *Physiochemical Characteristics.* The general physical and chemical characteristics of this inhibitor as prepared by Method 2 are as follows: It is a cream colored waxy ma-

[8] Tocantins, L. M., *Am. J. Physiol.*, 1945, **143**, 67.

TABLE II.
Effect of Human Lipid Inhibitor on Human Plasma Activated by Human Thromboplastin or by Human Thrombin.

Lipid inhibitor (mg)	Clotting time (sec.)					
	Undilut. thrombopl.*	Dil. thrombopl.*	Undilut. thrombin†		Dil. thrombin†	
			0 inc.	10 min. inc.	0 inc.	10 min. inc.
0	14	21	20	694	39	1408
5	117	337	19	580	37	960
10	350	1348	19	520	36	744
20	658	>24 hr	19	450	36	528
50	>24 hr					

* 0.1 cc plasma, 0.1 cc inhibitor solution (or 0.85% NaCl), 0.1 cc thromboplastin, 0.1 cc 0.02 M $CaCl_2$.
† 0.1 cc heated (56°C for 5 min.) plasma, 0.1 cc inhibitor solution (or 0.85% NaCl), 0.1 cc thrombin solution (0 or 10 min. incubation), 0.1 cc fresh plasma. Thrombin prepared by the method described by Eagle.[13]

terial, soluble readily in ether, methyl alcohol, chloroform, carbon tetrachloride, benzene, pyridine, glacial acetic acid and warm 95% ethyl alcohol. It is insoluble in acetone and cold 95% ethyl alcohol. The lipid inhibitor described by Chargaff[3] is only slightly soluble in cold pyridine and ether and is recrystallizable from methyl alcohol. Our inhibitor gives a negative Molisch test, and negative reactions to various qualitative protein tests (biuret, xanthoproteic, Acree-Rosenheim). It is not affected by oxidation or drying at room temperature. In the less pure state (Method 1), its potency can be increased by exposure to ultra-violet light. However, this property diminishes as purification proceeds, possibly indicating the removal of a coagulant impurity susceptible to the rays. That the inhibitor is still in an impure state is indicated by its melting point, which ranges from 182-200°C. In a 10% solution in 0.85% NaCl it has a pH of 6.0-6.1. It appears to have optimum activity at a pH of 7.0-7.3.

6. *Species Preferential Action.* Thromboplastins from human, rabbit and mouse brain are most active in the plasma of the corresponding species (Table III). Moreover, human lipid inhibitor is most effective against

TABLE III.
Comparison of the *in Vitro* and *in Vivo* Action of Thromboplastins and Inhibitors Prepared from Various Species.

	Effect *in vitro**			Effect *in vivo*† Thromboplastin activity (units per cc)
	Human plasma	Rabbit plasma	Mouse plasma	
	Clotting time in seconds			
1. (a) Human thromboplast.	14	14	15	120
(b) Human inhibitor " thromboplast.	164	93	18	120
2. (a) Rabbit thromboplast.	14	7	13	—
(b) Rabbit inhibitor " thromboplast.	122	37	32	—
3. (a) Mouse thromboplast.	72	22	9	260
(b) Mouse inhibitor " thromboplast.	1850	104	38	200

* (a) 0.1 cc plasma, 0.1 cc thromboplastin, 0.1 cc 0.85% NaCl, 0.1 cc 0.02 M $CaCl_2$. (b) 0.1 cc plasma, 0.1 cc thromboplastin, 0.1 cc inhibitor, 0.1 cc 0.02 M $CaCl_2$.
† (a) One unit is equivalent to the minimum lethal dose, or the minimum amount of thromboplastin solution which when injected intravenously will kill within 30 min., at least one and produce convulsions in at least one other, out of five 20-25 mice. Total volume of injected material 0.25 cc, injected within 3 sec.
(b) Inhibitor suspension 0.12 cc, injected intravenously 1-2 min. before injection of 0.12 cc of the thromboplastin solution.

human thromboplastin when tested in its own plasma, less effective when tested in rabbit plasma and almost ineffective when mouse plasma is used (Exp. 1, Table III). Rabbit inhibitor likewise seems to exert its greatest antithromboplastic effect in human plasma and least in mouse plasma (Exp. 2, Table III). Mouse inhibitor, however, exerts a greater effect against its own thromboplastin in human and rabbit plasma than in its own plasma (Exp. 3, Table III). This may indicate that a cofactor in plasma is necessary for the antithromboplastin action of the inhibitor. Human plasma would seem to have a greater amount of this cofactor than rabbit plasma, and rabbit plasma in turn more than mouse plasma.

Like Schneider[9] we have found that when an undiluted solution of human thromboplastin is injected intravenously into mice, the animals usually die from intravascular clotting within about 3 minutes after the injection. Most human brain thromboplastin solutions contain from 80 to 160 *in vivo* thromboplastin units per cc (Table III). When equal amounts of human lipid inhibitor and human thromboplastin are injected into mice intravenously, 1-2 minutes apart, the minimal lethal dose of the thromboplastin is unchanged. Mouse brain thromboplastins have contained from 160 to 260 *in vivo* units per cc of solution. If before an intravenous injection of the thromboplastin, an adequate amount of the mouse inhibitor is injected, the potency of the thromboplastin is reduced (Table III). Some mice are completely protected against weak mouse thromboplastin solutions by a preliminary injection (1-2 minutes before) of the lipid inhibitor. It seems, therefore, that we find *in vivo* the same species preferential activity of the inhibitor as *in vitro*. The human inhibitor cannot protect the mouse against an injection of human thromboplastin. The mouse inhibitor, however, can protect the mouse against an injection of its own thromboplastin.

7. *Anticoagulating Effect in vivo.* Lipid inhibitor solutions prepared from rabbit brain were injected intravenously into rabbits (600 mg per kg body weight). There were no apparent constitutional reactions, except for difficulty in checking bleeding from wounds made for collection of blood samples. In Chart 7 are 2 examples of a comparison of the results of such an injection with that of heparin (300 units, or 3 mg per kg body weight). One week was allowed to elapse between injections of the 2 anticoagulants and the same animals were used for both experiments. The doses were selected on the basis of the relative potency of the 2 anticoagulants *in vitro* against activated plasma (1 mg lipid inhibitor = ½ unit heparin). The curves demonstrate that though heparin may exert a greater anticoagulant action when dilute thromboplastin is used as test solution, its effect is dissipated within 1½ hours, while some evidence of activity of the lipid inhibitor is still detectable 5-6 hours after injection. Moreover, the lipid inhibitor seems to exert a greater action than heparin against the stronger thromboplastin solution. In the

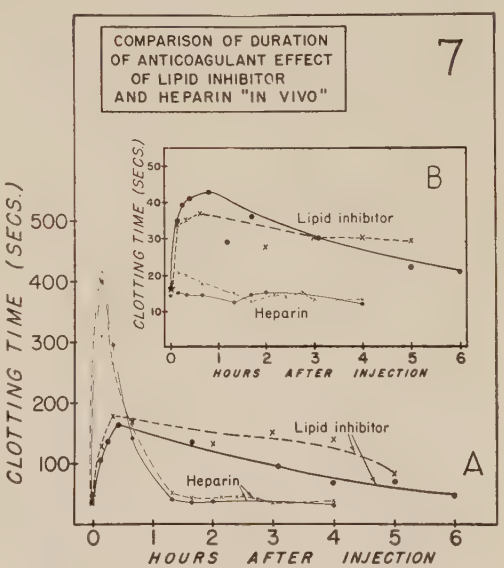


CHART 7.

Clotting times of two rabbits after intravenous injection of the lipid inhibitor or of heparin. A—Clotting times of 20 cu mm of blood (ear vein) to which 20 cu mm of a diluted solution of rabbit brain thromboplastin was added. B—Clotting times of 20 cu mm of blood to which 20 cu mm of an undiluted solution of the same thromboplastin was added. Readings on glass surfaces at 20°C.

[9] Schneider, C. L., *Am. J. Physiol.*, 1947, **149**, 123.

TABLE IV.
Comparison of Source, Potency, and Other Characteristics of Four Inhibitors of Blood Coagulation.

Anticoagulant:	Heparin (Toronto)	Chargaff 1937[3]	De Suto Nagy 1944[4,10]	Tocantins, Carroll and McBride 1948[14]
Source:	Beef lung	Sheep and pig brain	Pig spleen	Human brain
Chemical nature:	Sulfuric acid ester	Lipid	Protein lipid complex	Lipid
Behavior to heat:	Not destroyed by boiling	Not destroyed in boiling alcohol	Not destroyed in boiling alcohol	Destroyed after 10 min. 65-70°C
Mode of action:				
Antithromboplastin	Yes	—	Yes	Yes
Antithrombin	Yes	—	Yes	No
Antifibrinogen	No	—	No	No
Calcium binding	No	—	No	No
Concentration of solution:	0.005%			10%
Clotting time:				
Plasma + $CaCl_2$	250-350 sec.	120 sec.	348 sec.	250-350 sec.*
Plas. + anticoag. + $CaCl_2$	>24 hr	4920 "	>1800 "	>24 hr*
Plas. + thrombopl. + $CaCl_2$	12 sec.		9 "	12 sec.*
Plas. + thrombopl. + anticoag. + $CaCl_2$	81 "		9 "	280 " *
Source of plasma:	Man	Man	Dog	Man

* Figures quoted were obtained in tests carried out in glass tubes to make it possible to compare our findings with those of other workers.

quantities used, heparin does not seem to be *in vivo* as strongly antithromboplastic as the lipid inhibitor (Chart 7).

Comment. The lipid inhibitor of blood coagulation here reported has some similarities with that described by Chargaff,[3] but unlike it, our inhibitor is extracted in the cold, is heat labile and easily soluble in ether. It does not seem to be the same substance discussed by De Suto Nagy,[4,10] for it has widely different chemical properties and mode of action. Our inhibitor has greater potency than either of the other 2 substances (Table IV).

The fluidity of circulating blood seems to be maintained by a balance between anticoagulant and coagulant factors in the blood and surrounding tissues.[11] Whether or not the inhibitor here reported or a similar one play a role in aiding to maintain this fluidity remains to be clarified by further work. Thromboplastic substances liberated from damaged tissues and blood cells disrupt this equilibrium and initiate the changes (first phase of coagulation) which lead to clotting. The natural antithromboplastin of the plasma slows the development of clotting by reducing the amount of thromboplastin available for the activation of prothrombin. An antithromboplastin substance has been extracted from the plasma with methanol; when the methanol extract is added to the plasma, it enhances its antithromboplastic activity.[12] The lipid inhibitor here described is also soluble in methanol and has, furthermore, a range of thermolability similar to that of the natural antithromboplastin of the plasma.[7]

The necessity of a plasma cofactor for the development of the antithromboplastic effect of the lipid extract may explain why inhibitors and accelerators of coagulation may exist side by side in the tissues without neutralizing each other's action. The lipid inhibitor becomes an effective antagonist to its own thromboplastin, only in the presence of a plasma cofactor. Further observations on the nature of this cofactor and the species preferential activity of the lipid inhibitor are

[10] De Suto Nagy, G. J., *Am. J. Physiol.*, 1944, **141**, 338.

[11] Tocantins, L. M., *Blood*, 1947, **1**, 156.

[12] Carroll, R. T., and Tocantins, L. M., to be published.

being reported in greater detail elsewhere. The data already presented, however, serve to stress the necessity of avoiding the mixing of clotting reagents derived from different species. This is particularly applicable to thromboplastins and antithromboplastins, a precaution that has been stressed by us[7] and others.[13]

No direct correlation seems to exist between the effectiveness of heparin and the lipid inhibitor as tested *in vivo* and *in vitro*. While heparin is the stronger anticoagulant, the fact that it is rapidly excreted or inactivated *in vivo* has seriously limited its clinical usefulness. The longer duration of the effect of an intravenous injection of the lipid anticoagulant when compared with that of heparin seems to make the lipid more suitable in the anticoagulant therapy of thromboembolic disease.[14]

Summary. A heat labile lipid inhibitor of blood coagulation has been extracted from brain tissue, differing in potency and other respects from similar known anticoagulants. It has a pronounced antithromboplastic activity especially against homologous brain extracts in homologous plasma. Though the anticoagulant power of this lipid antithromboplastin *in vitro* is still less than that of heparin, it has a more lasting effect when injected intravenously than a solution of heparin of equivalent potency.

[13] Fantl, P., and Nance, M. A., *Med. J. Australia*, 1946, **2**, 125.

[14] Tocantins, L. M., Carroll, R. T., and McBride, T. G., *Fed. Proc.*, 1948, **7**, 125.

16409

Blood Volume and Sympathectomy in Hypertension.

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It has been observed that in hypertensive patients following sympathectomy there frequently is a return of blood pressure to the preoperative level after a brief period of relative hypotension. Since blood volume is an important factor in maintenance of the blood pressure level, it seemed advisable to investigate the possible changes in blood volume consequent to the postulated enlargement of the vascular bed produced by sympathectomy and to correlate these changes with those in the blood presure. Our results indicate that there are no consistent changes in total blood volume or in plasma volume during periods as long as 18 months after operation. Immediate postoperative reduction in red cell mass with concomitant increase in plasma volume occurred frequently and persisted in many cases. The postoperative prognosis in patients with low red cell mass preoperatively seemed to be poor.

Adequate postoperative determinations were available for 20 patients, 11 of whom were women and 9 men, ranging in age from 22 to 50 years. Follow-up observations ranged from 3 to 18 months. Thoracolumbar sympathectomy was done in all cases except one in which the transthoracic approach was used. Plasma volumes were measured photocolorimetrically with the T-1824 dye according to the procedures outlined by Gregersen.[1] Hematocrit readings were obtained by the Wintrobe method and these values were employed to calculate whole blood and red cell volumes. Standard values of 45 cc/kg were used for the plasma volumes of men, 40 cc/kg for women and 40 and 35 cc/kg for red cell volumes in men and women, respectively.

[1] Gregersen, M. I., *J. Lab. and Clin. Med.*, 1944, **29**, 1266.

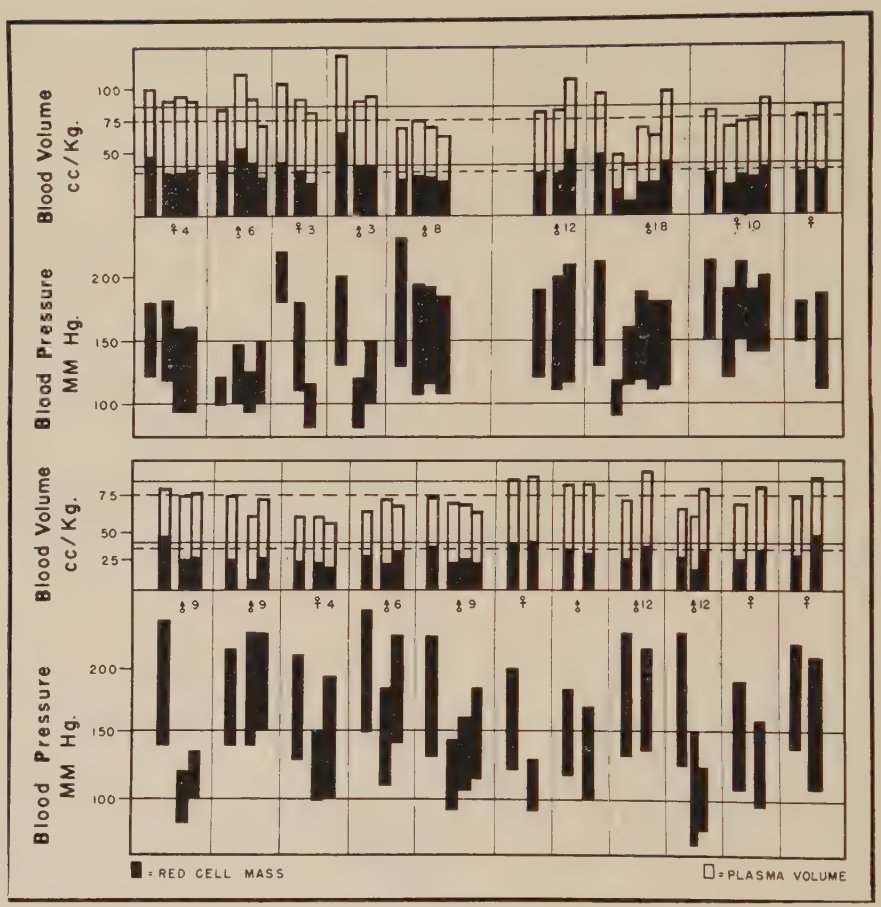

FIG. 1.

Relationship of plasma, red cell, and total blood volume to blood pressure. Each group of bars represents one patient and the first bar of each group represents the preoperative level. The shaded part of the blood volume bar represents red cell volume and the unshaded part is plasma volume. The small figures denote the duration of follow-up in months.

No consistent variation in blood volume from "normal" levels was observed preoperatively.[2,3] The results are shown in Fig. 1. In the first 4 patients there was close agreement between the post-operative drop in blood pressure and the changes in blood volume even for each individual determination. This would suggest that the blood volume change had a significant effect on the blood pressure or that the vascular tree was somewhat underfilled as a result of post-operative vasodilation.

In the next patient there was close agreement between the blood volume and blood pressure as the latter rose postoperatively and in the next case there was similar post-operative agreement until 18 months after operation when the blood volume returned to the preoperative level while the blood pressure was still significantly reduced. In the subsequent cases there was a sharp drop in blood pressure with little or no alteration in blood volume, as well as changes in blood volume with no change in blood pressure. Thus,

[2] Harris, A. W., and Gibson, J. G., Jr., *J. Clin. Invest.*, 1939, **18**, 527.

[3] Rowntree, L. G., and Brown, G. E., *The Volume of the Blood and Plasma in Health and Disease,* Philadelphia, W. B. Saunders & Co., 1929, p. 158.

the absence of obligate relationship is emphasized. There was also no correlation between postoperative blood volume and postural changes in blood pressure. These results are in agreement with those of Freis and Smithwick,[4] who studied 10 patients for 6 months postoperatively.

Immediately following operation there was a significant decrease in total red cell volume in the majority of patients (Fig. 2.). In many of these, return to standard levels was delayed for considerable periods postoperatively (Fig. 3). When the patients were grouped with regard to the manometric and symptomatic response to operation (Fig. 4), it was seen that the 5 patients who had exceptionally poor postoperative results all had low circulating red cell masses before operation whereas the 5 who had good results exhibited normal, or in 3 cases, elevated red cell masses preoperatively. There was a general tendency for patients with long standing or severe vascular disease to have low red cell masses and total blood volumes.[5]

The postoperative changes fall into several interesting groups. There was a general tendency for the red cell mass to drop immediately after the operation with a concomitant increase in plasma volume. These changes cannot be ascribed to bed rest alone, since prolonged bed rest results primarily in a decrease in plasma volume.[6] We believe that the decrease in red cell volume is a nonspecific effect related to postoperative blood loss;[4] this would suggest that more adequate blood replacement during operation might be indicated. The persistent low red cell levels are more difficult to explain. Schafer[7] reported a decrease in red cell mass in sympathectomized hypertensive dogs and in one patient with polycythemia vera and suggested that sympathectomy resulted in improved blood flow to the bone marrow and a consequent decrease in erythropoetic stimulus. Freis and Smithwick[4] also noted a tendency

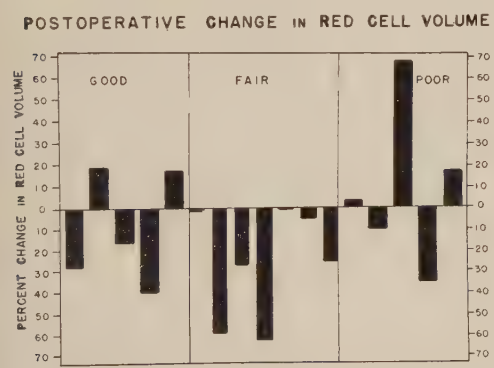

FIG. 2.

Changes in red cell volume approximately 2 weeks after sympathectomy. In this and in succeeding figures the patients are grouped with regard to symptomatic and manometric responses. Each bar represents one patient.

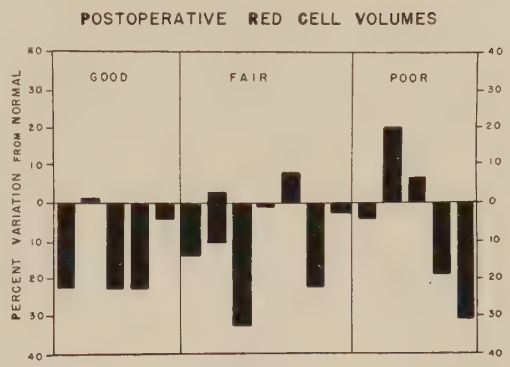

FIG. 3.

Red cell volumes at conclusion of experiments. See Fig. 1 for duration of follow-up periods.

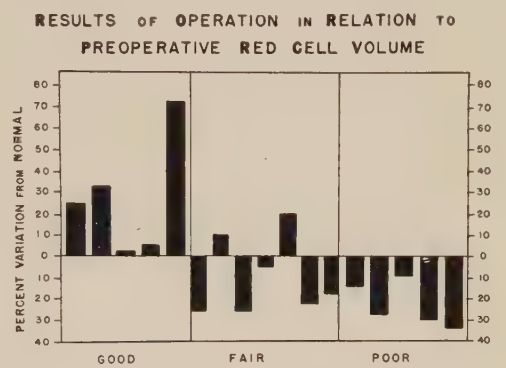

FIG. 4.

Relationship of preoperative red cell volume to operative response.

[4] Freis, E. D., and Smithwick, R. H., *Am. J. M. Sc.*, 1947, **214**, 363.

[5] Clark, J. H., Nelson, W., Lyons, C., Mayerson, H. S., and DeCamp, P., *Ann. Surg.*, 1947, **125**, 618.

[6] Taylor, H. L., Erickson, L., Henschel, A., and Keys, B., *Am. J. Physiol.*, 1945, **144**, 227.

[7] Schafer, P. W., *Ann. Surg.*, 1945, **122**, 1098.

for hematocrit values to remain low after sympathectomy but suggested that the change might be more apparent than real. They believe that sympathectomy, by altering the caliber of the smaller vessels, may cause redistribution of the red cell mass in the vascular system. This in turn would change the cell-plasma ratio in the larger vessels from which sampling is accomplished. Although this possibility cannot be excluded, it can hardly be of the magnitude and persistence required to explain our observations. In addition, any significant changes resulting from sympathectomy involve arterioles rather than capillaries and should not necessarily result in the shifting of large portions of the blood volume. The absence of any significant increase in blood volume after sympathectomy raises the question as to whether any widespread dilatation has actually occurred. A similar pronounced reduction in hematocrit was observed by Green[8] after surgical removal of a pheochromocytoma. The explanation of these observations is as yet obscure.

Although our series is admittedly small, it seems probable that patients with low red cell masses preoperatively can expect little from sympathectomy whereas if the red cell mass is elevated the prognosis is considerably better. Whether this is simply another way of saying that patients operated on early in the course of the disease do better postoperatively cannot be determined from these data.

Summary. Blood volume studies on 20 patients with hypertensive vascular disease before and after sympathectomy revealed no consistent long-term postoperative changes other than immediate decrease in the red cell mass with tendency toward delayed recovery. There was no consistent deviation from "normal" in the preoperative determination. Patients with low red cell masses preoperatively showed poor postoperative response and there was a general tendency for patients with long-term vascular disease to have low blood volume.

[8] Green, D. M., *J. A. M. A.*, 1946, **131**, 1260.

16410 P

Production of Alloxan Diabetes in the Dog.

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The discovery that a diabetic state could be induced in experimental animals by a single injection of alloxan opened a new field for the investigation of problems relating to the control of sugar metabolism. This technique has been applied and found successful in most of the ordinary species of laboratory animals. However, the results are not nearly so satisfactory in one of the more valuable experimental animals, the dog. Ordinarily, a satisfactory diabetic dog is obtained in only one out of 7 or 8 attempts. Doses of alloxan greater than 100 mg per kilo are frequently fatal, and smaller doses usually do not produce an appreciable and sustained elevation of the blood sugar. Failures in these larger laboratory animals is expensive and time consuming and makes a more effective method desirable.

The alloxan in varying dosages was injected directly into the abdominal aorta, and the hepatic, gastro-duodenal, and pancreatic arteries in an attempt to obtain a higher concentration of the drug in the pancreas with a smaller amount of the drug in the general circulation, thereby avoiding toxic effects on other organs. These attempts failed: only one in 8 dogs developed a moderate hyperglycemia and this effect did not persist longer than 2 weeks.

Kass and Waisbren[1] increased the per-

[1] Kass, E. H., and Waisbren, B. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 303.

TABLE I.
Fasting Blood Sugars.

Animal No.	Control	Days after alloxan administration* 1	2	3	6	7	8	10	11	12	13	14	15	19	Hyperglycemia
1	125	179		337				435	Insulin started				150	155	Sustained
2	126	250		362				421	Insulin started				210	320	"
3	78	325		127		178									Transitory
4†	181					262				152		166			"
5†	160					323	Insulin started			287		215			Sustained
6†	188					181				122		112			Transitory
7	140	600	died												Severe
8	140	583	"												"
9	100	750	"												"
10	120	370	Insulin started		190						175				Sustained

* Each dog received 75 mg per kilo of alloxan intravenously.
† These animals received part of dose subcutaneously, consequently did not develop severe or sustained diabetes.

centage of alloxan diabetes in rats by starving the animals for 48-60 hours before injecting the alloxan. A similar procedure has been applied to the dog and found to be successful.

Method. After maintaining the animals for three days on water only, 75 mg per kilo of body weight of alloxan in 5% solution was given intravenously as a single dose. Immediately following injection the animals were fed. If food was withheld for as long as 6 hours after receiving the drug, the animals would not ordinarily survive. Control blood sugars were taken at intervals thereafter. Specimens for blood sugar determinations were taken with the animals in a fasting state, food having been withheld for 12 hours. After diabetes had become established, it could be controlled with insulin.

Results. Of the 10 dogs injected, 7 developed a sustained or severe hyperglycemia and 3 developed a transitory hyperglycemia, as shown in Table I. Animals 11, 12, and 13 received part of their doses subcutaneously and this alteration of treatment is assumed to account for the less severe and transitory hyperglycemia observed. Animals 14, 15, and 16 received no food for 6 hours following injection, and they died on the second day after the administration of the drug. The greatly elevated blood sugar levels occurring in these 3 animals on the day following injection indicate that the islet cells were severely damaged with a resultant, sustained hyperglycemia had the animal survived. The diabetic animals were sacrificed for post-mortem examination after 45 days.

Discussion. The reasons for the effectiveness of the simple procedure are not entirely clear. Kass and Waisbren reported that the action of alloxan could be prevented by the injection of adrenalin just preceding or along with the alloxan, or by intravenous glucose administration 6 hours before the injection of alloxan. If the glucose were given one hour before the alloxan it would not prevent the development of the diabetic state. These data, together with the fact that starvation enhances the diabetogenic action of alloxan suggest that its action may in some way be related to the functional state of the glucose regulating mechanism. Lazarow[2] found that the hyperglycemic effect of alloxan could be prevented by the administration of glutathione and cysteine and he proposed that alloxan may act by inhibition of the sulfhydryl enzymatic system. He suggested that normally this system may be relatively deficient in the beta cells of the pancreatic islets. Starvation may further lower the sulfhydryl enzymatic system, and enhance the action of

2 Lazarow, Arnold, PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 441.

alloxan. These speculations require further investigation.

Conclusion. Alloxan diabetes can be produced in a high percentage of dogs by the simple expedient of starving the animals for 3 days prior to the injection of alloxan.

16411

Effect of Liver Extract on Growth of Rabbits.*

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The increasing use of rabbits in biochemical and nutritional investigations has led to further study in preparation of simplified diets for these animals. Wooley and Sebrell[1] described a purified diet supplemented only with pure vitamin preparations. Nearly normal growth was obtained for a limited period of time when this diet was fed.

In the present investigation we have studied the adequacy of diets for rabbits in which the vitamins were supplied in a purified form and also the effect of the addition of a 1:20 liver concentrate powder† to this diet.

Experimental. Rabbits of the New Zealand White breed were used in all experiments. The rabbits were weaned at 5 to 6 weeks of age at a weight of about 950 g and placed on the experimental diets. The change from the stock diet to the simplified diets was effected gradually over a period of three days after which time the new diet was accepted by all animals.

Preliminary observations were obtained with several diets to determine the best diet to use as a basis for comparison in a series of investigations on the nutritional requirements of rabbits. Results obtained with 3 of these diets, 2 of which contained 4% of the liver concentrate (rations 11 and 12), indicated that when liver concentrate was added to the diet an improved growth response was obtained. The average rate of growth of the rabbits which received the liver concentrate was significantly higher as determined by "analysis of variance" than for the rabbits fed ration 1, without the liver concentrate (P equals 0.02). The average rate of growth, during a 6 week experimental period, of the animals fed ration 1 was 175 g per week, while the average gains of those fed rations 11 and 12 containing liver concentrate were 226 and 209 g per week for the respective diets.

Rations 11 and 12 differed essentially only in the cellulose content. Variation in rate of growth was not as great on the 6% level of cellulose (ration 11) as on the 12% level (ration 12). The average gains, however, were not significantly different and in the statistical analysis the data obtained from rabbits on theses diets were treated as a single group.

To extend these observations weanling rabbits were fed a purified basal diet (ration 14) which was composed of casein 22%, peanut oil 8%, cerelose 53.5%, cellulose 12%, Salts IV[2] 4%, Vitamin A and D Concentrate (Nopco XX) 0.5%, mixed tocopherols 50 mg, choline 200 mg, niacin 20 mg, inositol 10 mg, pyridoxine 0.7 mg, thiamine 0.7 mg, riboflavin 0.7 mg, calcium pantothenate 1.5

* This work was supported in part by a grant from the Dow Chemical Company through the Texas A & M Research Foundation.

1 Wooley, J. G., and Sebrell, W. H., *J. Nutrition*, 1945, **29**, 191.

† Generously supplied by the Wilson Laboratories, Wilson and Co., Inc., Chicago, Ill.

2 Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

TABLE I.
Growth of Rabbits Fed Purified Diets and Diets Containing Liver Extract.

Experimental series No.	Diet No.	Diet	No. of animals	Growth in g per week
I	1	Purified diet	12	175
	11	" " + liver concentrate	6	226
	12	" " + " "	6	209
II	14	Purified diet	5*	107
	16	" " + liver concentrate	5	168
III	14	Purified diet†	9	107
	14A	" " + folic acid, biotin, *p*-aminobenzoic acid, and cystine	9	114
	14B	Diet 14A + liver concentrate	9	152

* One rabbit died after 4 weeks.

† In this series purified casein (Labco) was used and in Experiments I and II crude casein was used.

mg, and 0.075 mg of 2-methyl, 1, 4 naphthoquinone per 100 g of ration. The liver concentrate was added to diet 16 at a 4% level at the expense of the cerelose.

The average gain per week, over a 6 week period, of rabbits fed ration 14 was 107 g while that of the rabbits fed ration 16 which contained the liver concentrate was 168 g (Table I). This confirmed the preliminary observations that the addition of liver concentrate gives an increased growth response.

Three rabbits fed ration 14 had one or more onsets of severe diarrhea during the course of the experiment and one rabbit died from this cause. Post mortem examination revealed only a small amount of food in the gastrointestinal tract, liquefaction of fecal material and gaseous distention of the intestines and cecum. Microscopic examination of the fecal material from rabbits with diarrhea failed to reveal any significant presence of pathogenic organisms. Similar cases of diarrhea and death occurred in other experiments where the diet did not contain the liver concentrate.

Several of the rabbits kept on simplified diets which did not contain liver concentrate for 6 or more weeks suffered an abnormal loss of hair. It was doubtful in some cases whether or not this loss of hair was entirely spontaneous for it appeared to be due in some cases to the animals pulling and eating the hair from each other. A pronounced symmetrical alopecia occurred on both the dorsal and ventral aspects of the body and on the face and limbs of the animals fed ration 14. The hair coat of rabbits fed the liver concentrate diets appeared normal and healthy at all times.

Although recent studies have demonstrated that the rabbit synthesizes large amounts of pantothenic acid, riboflavin, folic acid and biotin,[3] it seemed desirable to test the effect on the rate of growth of supplementing the basal ration with folic acid and biotin since these two vitamins were not included in basal ration 14. *P*-aminobenzoic acid was included in the ration along with folic acid and biotin because of the possibility of synthesis of folic acid from this compound, and cystine was included because of the possibility of a combined deficiency of methionine and cystine as a consequence of using casein as the only protein in the ration. Three groups with 9 rabbits in each group were used in this experiment. One group received the basal ration (No. 14), the second group the basal ration plus the following supplements per 100 g of ration: folic acid (pteroylglutamic acid) 0.2 mg, biotin 10 μg, *p*-aminobenzoic acid 0.2 mg, and cystine 200 mg (ration No. 14A). The third group received ration No. 14A plus 4% of liver extract added at the expense of the cerelose. The animals were fed these diets for 7 weeks and the average rates of gain per week are shown in Table I. It can readily be seen that the addition of liver extract again resulted in an improved

[3] Olcese, Orlando, Pearson, P. B., and Schweigert, B. S., *J. Nutrition*, 1948, **38**, 577.

performance and that the addition of pteroylglutamic acid, biotin, *p*-aminobenzoic acid and cystine did not significantly improve the rate of growth. An analysis of variance showed that the addition of liver extract significantly increased the rate of growth as compared to when the basal diet was fed ($P = <.01$) and also as compared to when the supplemental diet (No. 14A) was fed ($P = <.05$).

Hemoglobin determinations were run after the animals had been on experiment for 5 weeks and no difference in the hemoglobin levels was observed for the groups receiving diets with or without liver extract.

Discussion. The results of these experiments demonstrate an improved performance in the rate of growth and general condition of health when rabbits are fed a diet containing a 1:20 liver concentrate. The data suggest that a water soluble concentrated liver extract, such as that used in this study, contains an unidentified factor or factors necessary for optimum growth and the general well being of the rabbit. It is also concluded that *p*-aminobenzoic acid, pteroylglutamic acid and biotin are not dietary essentials for the rabbit.

It has been reported that the ingestion of liver and various liver extracts results in improved performance with other species when the diets used were apparently adequate in all known nutrients. Schweigert *et al.*[4] reported increased growth rates with the cotton rat when fed liver extract. Recent work with the cotton rat[5] indicates that pteroylglutamic acid and/or biotin do not give the response given by the liver concentrate. Improved lactation in rats has been reported when pork or beef liver was added to a ration supplemented with minerals, fatty acids, and all known vitamins.[6] An increase in the percentage of young surviving the lactation period and small increases in the rate of growth were obtained when liver or liver concentrates were added to diets containing 10 B vitamins.[7] Various liver concentrates have been shown to markedly improve the performance of monkeys fed purified diets over long periods, particularly when the animals have been subjected to other nutritional deficiencies.[8,9] Similarly, liver concentrates contain factors not identical with known nutrients which are essential for the mink and fox.[10,11] These examples, as well as the observations reported here, suggest that liver and liver concentrates contain factors which are necessary for several species and the need for these factors becomes evident with the use of a variety of experimental criteria. The similarity and identity of these factors remain to be determined.

It is rather unlikely that the liver concentrate would contribute factors which would be supplementary to the protein portion of the diet. The 22 per cent level of casein and additional cystine is generally considered adequate for most species of animals.

Summary. The addition of 4% 1:20 liver concentrate to purified rations increased the rate of growth and aided in the maintenance of a general healthy appearance of rabbits. The improved performance noted when liver extract was added is apparently not attributable to known nutrients including folic acid, biotin, *p*-aminobenzoic acid and cystine. The results indicate that liver contains an unidentified factor essential for the maximum rate of growth of the rabbit.

[4] Schweigert, B. S., Shaw, J. H., Phillips, P. H., and Elvehjem, C. A., *J. Nutrition*, 1945, **29**, 405.

[5] Schweigert, B. S., unpublished data.

[6] Spitzer, R. R., and Phillips, P. H., *Fed. Proc.*, 1947, **6**, 422.

[7] Sporn, E. M., Ruegamer, W. R., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 5.

[8] Cooperman, J. M., Elvehjem, C. A., McCall, K. B., and Ruegamer, W. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 92.

[9] Ruegamer, W. R., Cooperman, J. M., Sporn, E. M., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **167**, 861.

[10] Schaefer, A. E., Whitehair, C. K., and Elvehjem, C. A., *J. Nutrition*, 1948, **35**, 147.

[11] Schaefer, A. E., Tove, S. B., Whitehair, C. K., and Elvehjem, C. A., *J. Nutrition*, 1948, **35**, 157; *Z. f. Vitaminforschung*, 1947, **19**, 12.

16412

Relation of Anemia and Hemorrhagic Shock to Experimental Ulcer Production.*

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The occurrence of acute ulcers in the upper gastrointestinal tract of humans has been reported by several authors[1-3] as sequelae of many diverse clinical conditions such as extensive surgical procedures, burns, fractures, diabetic acidosis, freezing, severe heart disease, and other grave systemic disorders. They noted that all of these cases had as a common denominator the factor of shock. It is our purpose to indicate some observations on the effect of anemia and post-hemorrhagic shock on the histamine-provoked ulcer in dogs.

Method. Fifty-one healthy dewormed dogs of medium size from 1 to 5 years of age were used in these experiments. The stimulus for ulcer production was provided by a daily intramuscular injection of 30 mg of histamine-base in beeswax after the method described by Code and Varco.[4] Feed pans were removed in the evening at the time of injection and the animals were not fed again until morning. Those animals not succumbing to ulcer formation were sacrificed 12 hours after the last histamine injection by intravenous sodium pentobarbital.

Experiment I. Fifteen dogs were bled acutely from the femoral artery or vein until an average of 42% of their estimated blood volume was removed. The withdrawal of this volume of blood uniformly leads to blood pressures of shock level. Histamine-in-wax injection was then started the first or second day following the bleeding. Six of these animals as indicated in Table I were bled under pentobarbital anesthesia with continuous kymographic recording of their blood pressure from the carotid artery. In these 6 dogs the blood pressure was maintained below 50 mm of mercury for periods from 30 to 55 minutes and then allowed to rise or was elevated by normal saline intravenously. The remaining 9 dogs were bled to shock level without anesthesia and without blood pressure recordings. Then quantities of saline solution varying from none to an amount equal to the volume of the blood withdrawn were injected intravenously as indicated in Table I. The period of shock in these dogs was relatively shorter than the above-mentioned 6 bled under anesthesia inasmuch as the saline intravenously was started immediately following the finish of rapid continuous blood withdrawal from the femoral vessels. All dogs in Experiment I had blood samples drawn for hemoglobin, hematocrit, and total protein levels before bleeding and at daily intervals thereafter. In those dogs in which the blood volume was not actually measured by the dye method, we used 10%[5] of their body weight as our criterion for estimating the total circulating blood volume. These above-mentioned differences in procedures are indicated in Table I.

Experiment II. In 13 dogs a chronic anemia without shock was produced by removing small quantities of blood (100-150 cc) not oftener than every 24 hours over a period of from 5 to 30 days. Observations were made also on hemoglobin, hematocrit,

* Supported by a grant from the Surgical Study Section of the United States Public Health Service, the John and Mary R. Markle Foundation and the Augustus L. Searle Fund for Surgical Research.

† National Cancer Trainee.

1 Penner, A., and Bernheim, A. C., *Arch. Path.*, 1939, **28**, 129.

2 Meyer, J., and Saphir, O., *Am. J. Dig. Dis.*, 1943, **10**, 28.

3 Boles, R. S., Riggs, Helena A., and Griffiths, J. O., *Am. J. Dig. Dis.*, 1939, **6**, 632.

4 Code, C. F., and Varco, R. L., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 475.

5 Hematologic Data of Normal Adult Dog, *J. A. M. A.*, 1946, **132**, 1103.

TABLE I.
Production of Ulcer in Dogs Following Acute Hemorrhage with Shock.
(30 mg histamine-base in beeswax daily).

Dog No.	% bl. vol. withdrawn	Duration of shock min.	cc-saline replaced	12 hr after bleeding: g Hbg.	12 hr after bleeding: cc/100 Hct.	No. hist. inject.	Results
954*	30	42	—	13.1	41	5	Perf. duod. ulcer. Gastric erosions
968*	34	55	250	10.4	32	4	Negative G. I. T.
377	36	30	100	8.0	31	5	" " " "
386	53	30	150	10.3	35	5	Two duod. ulcers
438	34	30	400	7.2	31	5	Petechial hemorrhages in antrum
439	28	30	450	9.7	33	5	Six small duod. erosions
889	57	—	—	—	—	1	Prepyloric ulcers. Multiple antral petechia
892	52	—	—	8.4	29	5	Negative G. I. T.
899	29	—	—	15.1	48	5	Perforated duod. ulcer
906	53	—	500	9.6	32	4	" " "
917	72	—	850	6.6	22	8	Multiple antral erosions
935	48	—	250	10.0	34	4	Antral ulcer and erosions
946	14	—	—	9	35	5	Two large duod. ulcers
947	51	—	225	8.4	28	5	Negative G. I. T.
959*	44	—	—	8.5	34	6	Perforated gastric ulcer

* Blood volume measured before and after bleeding.

No. of dogs injected	15
" " " with ulcer	8
" " " with erosions	3
" " " with perf. ulcers	4

TABLE II.
Production of Ulcer in Dogs with Chronic Anemia (No Shock).
(30 mg histamine-base in beeswax daily).

Dog No.	Init. wt, lb	Total amt. bl. withdrawn, cc	Bleeding period, days	At onset hist. inj.: Hbg., g	At onset hist. inj.: Hct., cc/100 cc	No. inj. hist.	Results
900	36	1050	7	11	32	8	Neg. G. I. T.
977	41	1135	9	9.6	31	5	" " " "
988	25	780	6	6.6	23	5	" " " "
10	30	1500	9	5.2	21	5	" " " "
9	35	1250	14	7.2	27	5	Single duod. ulcer
45	28	2500	30	6.1	31	5	Duod. and jejunal ulcers
53	39	1600	9	7.2	27	5	Duod. ulcers
99	30	1150	8	6.1	24	5	Shallow duod. ulcer
138	28	1300	10	6.5	25	5	Neg. G. I. T.
372	30	800	8	6.8	29	3	Neg. G. I. T. Sev. pneumonia
373	28	1035	8	6.6	24	5	Neg. G. I. T.
397	28	950	5	6.6	24	5	" " " "
404	28	800	5	5.8	24	5	" " " "

No. of dogs injected	13
" " " negative	9
" " " with ulcers	4

and total protein levels before bleeding and daily thereafter. Blood pressures before and after bleeding were recorded by means of an exteriorized carotid artery loop in one dog and by femoral artery punctures in another.

Experiment III. Seven dogs were bled to shock level under sodium pentobarbital anesthesia with continuous blood pressure tracings. In 5 of these animals the blood pressure was maintained below 50 mm mercury for at least 30 to 40 minutes, and in the other 2 dogs the period of shock was shortened to 10 to 12 minutes. Then all dogs were given their own citrated blood back intravenously. Two and

TABLE III.
Production of Ulcer in Dogs Subjected to Shock (No Anemia).
(30 mg histamine-base in beeswax).

Dog No.	Wt	Duration of shock, min.	No. hist. injections	After shock: g Hbg.	After shock: Hct. cc/100 cc	Results
971	47	less than 10	5	12.8	43	Negative G. I. T.
995	40	40	6	14.9	49	Duodenal ulcer
1	39	12	6	16.6	58	Negative G. I. T.
378	20	30	5	11.0	37	Perforated duod. ulcer
383	29	30	5	11.0	45	Negative G. I. T.
436	18	30	3	16.0	51	Penetrating antral ulcer, duod. ulcers
437	28	30	5	14.4	46	Negative G. I. T.

No. of dogs tested 7
" " " with ulcer 3
" " " with perforated ulcer 1

TABLE IV.
Production of Ulcer in Normal Dogs.
(30 mg histamine-base in beeswax daily.)
Control Series.

No. of dogs	No. hist. injections	No. dogs with: Ulcer	No. dogs with: Erosion
16	5	2	1

one-half g of citrate was the maximum amount given to any dog.

As additional controls for this experiment, 2 animals were anesthetized and given an equal quantity of sodium citrate intravenously and a third dog was anesthetized and a laparotomy was performed. These 3 animals were then started on histamine-in-wax injections the following day and when sacrificed after 5 consecutive histamine injections all 3 had normal gastrointestinal tracts. In addition, 13 dogs of comparable size and age were given 5 to 6 consecutive daily injections of histamine-in-wax and sacrificed as controls. The data for this control group are listed in Table IV.

Results. The incidence of ulcerations in Experiments I, II, and III is shown in Tables I, II, and III, respectively. It is apparent that the dogs subjected to both anemia and shock were the most susceptible to ulcer formation. Inspection of Tables I and III would seem to indicate that the duration of shock within the limits tested in these experiments does not make a great deal of difference in ulcer incidence, a relatively short insult being as effective in abetting the ulcer diathesis as one lasting 30 to 40 minutes. Nor does the factor of saline replacement seem to affect the ulcer diathesis. The hemoglobin and hematocrit values shown in Table I are those taken approximately 12 hours after bleeding and subsequent determinations for most all of the dogs showed a progressive fall in the hemoglobin values and hematocrit levels for the first 3 to 4 days after bleeding. Moreover, inspection of these values suggests that the dogs having the greatest reduction in hemoglobin levels and hematocrit values had the least severe ulcers. This fact naturally suggested an attempt to separate the effects of the factors of anemia and of shock. In Experiment II we did not observe any blood pressure values to fall below 90 mm Hg so that we have anemia production uncomplicated by shock. Here the incidence of ulcers was somewhat higher than that observed in the control group of dogs (Table IV). However, in Experiment III, where hemorrhagic shock was produced by bleeding and then the resultant anemia corrected by giving the animals their own blood back, the incidence of ulcer approached that seen in Experiment I, indicating that of the 2 factors shock has the

greater effect in abetting the ulcer susceptibility.

Discussion. The ulcer abetting effect of shock is amply corroborated by both experimental and clinical evidence. Moon[6] lists gastric erosions and ulcers as a pathological feature of shock of any kind. Price[7] *et al.* in their studies on post-hemorrhagic shock observed subserous and submucous hemorrhages with bleeding into the lumen of the gastrointestinal tract in occasional dogs dying without therapy. Blalock[8] has observed the same gastrointestinal tract findings in his dogs following post-hemorrhagic shock.

The above-mentioned investigators and others agree that the characteristic physiologic responses to a large blood loss in surviving animals are anemia, hemodilution, and vasoconstriction. Our observations indicate that the factors of anemia and hemodilution are common to the dogs in both Exp. I and II, yet there is a pronounced difference in the ulcer incidence indicating to us that the ulcer abetting effect of post-hemorrhagic shock is a vascular one, namely, vasoconstriction. Klemperer[9] *et al.* in studying the gastrointestinal manifestations of shock in humans has claimed to be able to reproduce in animals all of the pathology seen in their autopsy material by causing visceral vasospasm by repeated intraperitoneal injection of epinephrine. Likewise the potent ulcer abetting effects of adrenalin-in-beeswax and pitressin-in-beeswax have been studied in this laboratory by Baronofsky and Wangensteen.[10] It was their conclusion that the chronic arterial spasm invoked by epinephrine or pitressin produces local areas of anemic gastric mucosa which then became susceptible to the acid-peptic activity of the gastric juice. A somewhat analogous condition also studied in this laboratory is the production of ulcers and erosions by plugging the end vessels of the upper gastrointestinal tract with the intravenous injection of fat.[10] Knisely[11] *et al.* have demonstrated in healthy unanesthetized human blood donors the intense vasoconstriction that occurs in the arteries, arterioles and capillaries of the bulbar conjunctiva following a moderate blood loss. Reasoning from the demonstrated role of hemoconcentration[12] in aiding and abetting the ulcer diathesis, it might be expected that hemodilution would have a potent analogous effect, but such does not appear to be true in our experience, for the dogs in Exp. II show a fairly uniform degree of hemodilution without a striking increase in their ulcer incidence.

Conclusions. The factors of post-hemorrhagic anemia and shock are evaluated in relation to their effect on the histamine-provoked ulcer in dogs. Our evidence indicates that anemia and hemodilution occurring without shock is a mild ulcer abetting factor. However, post-hemorrhagic shock, even of relatively brief duration aids and abets the ulcer susceptibility most likely through its vasoconstrictor effect on the small blood vessels with resultant localized anemic areas in the mucosa.

The combined effects of post-hemorrhagic anemia and shock on the ulcer diathesis are synergistic in that together they give a higher incidence of histamine-provoked ulcer than does either alone.

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6 Moon, V. H., *Shock: Its Dynamics, Occurrence, and Management*, 1942, Lea and Febiger, Philadelphia.

7 Price, P. B., Hanlon, C. R., Longmire, W. P., and Metcalf, W., *Johns Hopkins Hospital Bull.*, 1941, **69**, 327.

8 Blalock, A., *Arch. Surg.*, 1934, **29**, 837.

9 Klemperer, P., Penner, A., and Bernheim, A., *Am. J. Dig. Dis.*, 1940, **7**, 410.

10 Baronofsky, I., and Wangensteen, O. H., *Bull. Am. Coll. of Surgeons*, February, 1945.

11 Knisely, M. H., Bloch, E. H., Eliot, T. S., and Warner, L., *Science*, 1947, **106**, 431.

12 Friesen, S. R., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 81.

16413

Effect of Age on Histamine-Induced Ulcer in Dogs.*

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The observation was made in the course of other studies that rather frequently obviously senile dogs given histamine-in-beeswax injections develop severe peptic ulcers rapidly.

Method. Forty-seven dewormed healthy mongrel dogs were divided on the basis of their age into 3 groups. All dogs were subjected to histamine-in-beeswax injection after the method of Code and Varco.[1] Except where noted otherwise, all animals received a daily intramuscular injection of 30 mg of histamine-base-in-wax in the evening. At the time of the injection food pans were removed until the following morning. Unless the dogs died they were sacrificed by intravenous sodium pentothal 12 hours after the last histamine injection.

Group I. Senile dogs: consisted of 14 animals 10 years old or older ranging in weight from 17 to 66 lbs.

Group II. Mature dogs: consisted of 13 animals of comparable size from 1½ to 5 years of age.

Group III. Pups: consisted of 20 animals from 3 to 8 months of age and ranging in weight from 8 to 22 lbs.

All animals were fed a stock diet of vitamin enriched dog biscuit kibbles supplemented by daily fresh horse meat and milk. In addition the animals in Group I were fed the above diet plus a daily supplement of vitamins‡ for varying periods up to 6 weeks before histamine stimulation was begun in order to assay the effects of improved nutrition.

In determining the ages of animals, we obtained where possible the actual ages from the original owners. In addition no dogs were used unless one of us (C.W.L.) familiar with the veterinary literature concerning canine ageing and two experienced animal caretakers unanimously agreed on a dog's approximate age, which was then recorded before the experiment began. The changes associated with canine ageing from birth to senile decay with particular emphasis on the teeth have been described in detail by Kirk,[2] by Sisson,[3] by Garbutt,[4] and by Ellenberger and Baum.[5]

All dogs had a blood sample drawn initially for a hemoglobin and hematocrit determination. Also at least once while on histamine-in-wax stimulation each dog had a fasting sample aspirated from the stomach to determine the free and total acid levels.

Results. In Group I, consisting of 14 senile dogs given a daily intramuscular injection of 30 mg of histamine base in beeswax for 5 consecutive days or less, 9 dogs showed typical gastric or duodenal ulcers. Also within this period of 5 days, 4 of these 9 animals with ulcer died of a perforation. Four additional dogs in this group showed multiple gastroduodenal erosions. Only 1 animal in this age group had a negative gastrointestinal tract at the time of sacrifice.

In Group II consisting of 13 dogs of mature age given the same dose of histamine in beeswax intramuscularly for 5 days and then sacrificed, 2 animals showed a duodenal ulcer and 1 showed a single duodenal erosion. The other 10 dogs had negative gastrointestinal tracts.

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† National Cancer Trainee.

1 Code, C. F., and Varco, R. L., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 475.

‡ Vit. A, 10,000 units, 100 mg ascorbic acid, Vit. D, 1,000 units, 4 g brewer's yeast, daily to each dog.

2 Kirk, H., *The Veterinary Rec.*, 1945, **57**, 212.

3 Sisson, S., *The Anatomy of the Domestic Animals*, W. B. Saunders Co. 1914.

4 Garbutt, R. J., *Diseases and Surgery of the Dog*, Orange Judd Publishing Co., Inc., 1938, New York, N. Y.

5 Ellenberger, W., and Baum, H., *Anatomie des Hundes*, 1891, Paul Parey, Berlin.

TABLE I.
Ulcer Production in Relation to Dog Age.
(A daily intramuscular histamine-in-beeswax injection for 5 days.)

Age range (yrs)	Dose histamine base—mg/day	No. of dogs	No. ulcer and/or erosion	%
Over 10	30	14	13*	93
1½ to 5	30	13	3	23
Less 1	30	10	1	10
Less 1	15	10	1	10

* 4 dogs died of perforated ulcer.

When daily intramuscular injections of 30 mg of histamine-base-in-beeswax are given to normal healthy dogs of medium age, 40 days (or an average of 23 days) are necessary to produce ulcer with regularity.[6]

Group III consists of 20 pups of which 10 were given 30 mg of histamine-base-in-wax daily as in the above groups and 10 were given 15 mg of the histamine base. Although the body weights of these pups averaged ½ to ⅓ of the weights of the mature and senile dogs, only 2 pups out of the 20 developed an ulcer. The other 18 had negative gastrointestinal tracts at sacrifice. These results are summarized in Table I.

Because of the strong evidence suggesting a vascular etiology for peptic ulcers occurring for the first time in old patients, we made a careful search in these animals for arteriosclerosis but found none grossly or microscopically. We did, however, find microscopic evidence of degenerative changes in practically all dogs in Group I from whom satisfactory sections were obtained. The spleen showed the most consistent changes with senile atrophy manifested by a decrease to virtual disappearance of the lymphoid follicles and a relative increase in prominence and quantity of the trabeculae due to shrinkage in the size of the spleen. The other organ showing the most consistent changes was the liver which showed varying changes from severe chronic passive congestion with central hepatic atrophy (Dog. No. 86) to severe fatty infiltration (Dog No. 2). The severity of the liver changes did not appear to be correlated with the severity of the peptic ulceration in our series of dogs but was correlated with the time the dogs were fed stock diet before histamine was started. As would be expected the senile dogs showed a relative anorexia, eating much less than the younger animals in proportion to size. All animals exhibit anorexia coincidental with development of an ulcer on histamine stimulation.

The fresh stomach weights of all sacrificed animals were recorded at autopsy and when this weight was plotted against the dogs' body weight on a graph the animals again divide themselves into the same 3 groups as on the basis of the age with pups having the heaviest stomach weights in proportion to body weight and the senile dogs the lightest with the weights of the mature dogs' stomachs lying between. We take this finding as corroboration of the correctness of our criteria for judging age.

Only 1 dog showed a borderline anemia in Group I (10.3 g with a hematocrit of 34). This animal was fed a supplemented diet for 6 weeks before histamine injections were started but died after the fifth injection with a perforated duodenal ulcer.

Discussion. It is of interest that in examining the stomachs and duodenums of over 900 healthy dogs, Ivy[7] found only one animal with a spontaneous ulcer. This stomach, he states, was taken from an "old emaciated dog." We have no direct proof that some of our senile dogs did not have an ulcer before we gave them histamine except to say that even in old dogs in our own experience spontaneous ulcer has not been observed.

It is likewise of interest to note that the pups showed a greater resistance to ulcer formation in spite of the fact that on the basis

[6] Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg., Gyn., and Obst.*, 1942, **75**, 170.

[7] Ivy, A. C., *Arch. Int. Med.*, 1920, **25**, 6.

of weight they were given a considerably larger dose of histamine.

Similar gradations of susceptibility in relation to age have been reported for other conditions such as anoxia (Glass[8] *et al.*, Chang[9]) for cardiac sub-endothelial hemorrhages (Visscher[10] *et al.*), for myocardial lesions from digitalis (Barnes[11] *et al.*) and for certain drugs (Bastedo).[12] Moreover, there is evidence[13] that with advancing years the autonomic nervous mechanisms for the control of homeostasis become less efficient resulting in a vagus preponderance in old age.

The senile dogs in this series were 10 years old or older, which age, according to veterinarians[2] is equivalent to a human age of 70 years. The occurrence of peptic ulcers for the first time in humans in this age is not unusual. Klingenstein[14] observed that over one-half of the patients over 50 with proved gastroduodenal ulcer had the first onset of symptoms past the age of 50. Likewise Meyer and Saphir[15] have pointed out that old people are likely to develop acute ulcers and succumb to hemorrhage and perforation even with very low acid levels especially following major surgery, febrile disease, or cardiac disease. These clinical observations would suggest that in the genesis of ulcers, particularly in the aged, factors that have to do with mucosal resistance to digestion are at least as important as acid-pepsin levels. Much the same seems to hold true for these dogs inasmuch as the fasting gastric acidities of aspirated juice while on histamine-in-beeswax stimulation were the same in Groups I, II, and III. (Range 60-100° free acid for all groups).

The present study is of practical importance for those investigators studying the ulcer problem experimentally for it indicates that in using the histamine-in-beeswax technic, or more than likely any other ulcer-producing method, one should compare only dogs of the same approximate age.

Conclusion. There is a gradation on the basis of age in susceptibility to histamine-induced ulcer in the upper gastrointestinal tract of dogs. The oldest dogs exhibit the greatest incidence of ulcers and the pups from 3 to 8 months show the greatest resistance with the dogs of mature age intermediate in their susceptibility to ulcer formation.

8 Glass, H. G., Snyder, F. F., and Webster, E., *Am. J., Physiol.*, 1944, **140**, 609.

9 Chang, T. T., *Luftfahrtmedizin*, 1937-38, **2**, 239.

10 Visscher, M. B., and Henschel, A., *Am. Heart J.*, 1945, **30**, 592.

11 Dearing, W. H., Barnes, A. R., and Essex, H. E., *Am. Heart J.*, 1943, **25**, 648.

12 Bastedo, W. A., *Materia Medica, Pharmacology and Therapeutics*, 4th Edition, 1937, W. B. Saunders Co., Philadelphia.

13 White, J. C., and Smithwick, R. H., *The Autonomic Nervous System*, 1945, p. 68, The Macmillan Co., New York.

14 Klingenstein, P., *J. Mt. Sinai Hosp.*, New York, 1940-41, **7**, 432.

15 Meyer, J., and Saphir, O., *Am. J. Dig. Dis.*, 1943, **10**, 28.

16414

Aminoaciduria in Progressive Muscular Dystrophy.*

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In view of the wasting of muscle which is characteristic of progressive muscular dystrophy, it seemed probable that there might be an increased urinary excretion of minor nitrogenous products in addition to creatine, *e.g.*, free amino acids. On studying the urine from patients suffering from progressive muscular dystrophy we found that there was a generalized aminoaciduria characterized by

* Communication No. 129.

the excretion of a larger number of amino acids than is normally the case, accompanied in many instances by an excessive excretion of amino nitrogen.

Meldolesi[1] studied a number of cases of progressive muscular dystrophy and reported a decreased urinary excretion of nitrogen. However, results of recent studies by Milhorat and Tosconi,[2] and Shank, *et al.*[3] of nitrogen metabolism in progressive muscular dystrophy have failed to demonstrate any significant abnormality. It seems evident, therefore, that any alteration in nitrogen metabolism is not apparent in the measurement of total nitrogen excretion.

The use of partition chromatography to determine the distribution and excretion of urinary amino acids has been developed by Dent.[4] In a study of normal humans he usually found glycine and alanine and occasionally one or two other amino acids. He applied this procedure to the determination of abnormalities in several patients with Fanconi syndrome. In a recent paper,[5] he reported more fully on Fanconi syndrome, in which he found a large number of amino acids and an increased level of amino nitrogen. In 2 of the patients, amino nitrogen excretion was not excessively high but a greater number of amino acids were being excreted.

We have applied this technique in a study of 32 samples** of urine which were obtained from patients who had been diagnosed as suffering from progressive muscular dystrophy or related conditions. A portion of these were obtained by one or more voidings but about one-half represented 24-hour collections of urine. As indicated in Table I some of these patients were receiving therapy. The general diagnosis and an indication of the severity of the condition are given for each patient.

The urines were chromatographed on filter paper employing the strip technique essentially as has been described by Dent.[4] An aliquot of 0.025 ml was placed at a starting point on the strip. The adjacent end of the strip was placed in the solvent and remained there for 15 to 23 hours. The strip was then air-dried, sprayed with a solution of ninhydrin and heated to 100°C for 15 minutes. Colored bands were observed at the points where amino acids were to be found on the strip. Solvents employed were water-saturated solutions of phenol, butanol and a 50-50 mixture of lutidine and collidine. In many cases, strips were run in duplicate with satisfactory correspondence being obtained.

Two methods were used for evaluating the developed paper strips. An arbitrary notation was devised, in which strips which showed a normal number of colored bands were designated as "0"; those which showed a greater number or an unusual distribution were designated by "1"; and strips which showed an excessive number or very unusual distribution were designated as "2." The results of this general analysis agreed with Table I where practically all of the pathological urines indicated excessive amino-acid excretions when compared with normal urines. In addition to this general description, a numerical value was used which was arbitrarily set up as follows: The color intensity of a given band on the strip was visually estimated from "1" to "5" where "1" represented the faintest band observable and "5" represented an intensely colored band. If the color intensity of each band is multiplied by its width in cm, the sum of these products for a given solvent will be designated the *dw* value. It is felt that this *dw* value, although entirely arbitrary, gives a useful estimate of the dispersion and concentration of the individual amino acids appearing as bands on the paper strip. A normal human urine gives *dw* values less than 6 or 8. As may be seen from the data recorded in Table I, in most cases the *dw* values for the patients with progressive muscular dystrophy were greater than 10 and in some

[1] Meldolesi, G., *Policlinico, Sez. Prat.*, 1936, **43**, 1187.

[2] Milhorat, A. T., and Toscani, V., *Arch. Neurol. Psychiat.*, 1939, **41**, 1130.

[3] Shank, R. E., Gilder, H., and Hoagland, C. L., *Arch. Neurol. Psychiat.*, 1944, **52**, 431.

[4] Dent, C. E., *Lancet*, 1946, **2**, 637.

[5] Dent, C. E., *Biochem. J.*, 1947, **41**, 240.

** These samples were obtained through the courtesy of Dr. A. T. Milhorat of the New York Hospital and Drs. K. E. Mason and L. J. Filer, Jr., of Strong Memorial Hospital, Rochester.

TABLE I.
Urinary Excretion of Amino Acids in Progressive Muscular Dystrophy and Related Conditions.

Diagnosis	No. of patient	Creatine† excretion	*dw* value	Amino-N mg/ml	No. of spots	Degree of aminoaciduria
Progressive Muscular Dystrophy						
Pseudohypertrophic Type						
Far advanced	201*	+	23	1.14	15	High
Advanced	189	+	22	1.39	14	"
	195*	+	21	0.49	9	Moderately high
Moderately Advanced	159*	—	4	0.23	8‡	Normal
	211	+	16	0.29	12	High
	229	+	12	0.42	10	Moderately high
	230	+	11	0.43	10	" "
Early	168*	—	3	0.59	8‡	Normal
Facio-Scapulo-Humeral Type						
Advanced	155*	—	11	0.49	8‡	Moderately high
Moderate	167	+	26	0.85	14	High
Early	157*	—	8	0.39	11‡	Moderately high
	162*	—	7	1.03	10‡	" "
	165	—	5	0.22	8	Normal
Other Types						
Far advanced	197*	+	25	0.51	11	High
Advanced	163*	—	20	1.02	18‡	"
	161	—	21	0.44	14	"
	156*	—	8	0.30	12‡	"
	158	+	15	0.58	9	Moderately high
	154*	—	22	0.48	6‡	" "
Moderately advanced	152	—	22	0.71	13	High
	153	—	16	0.62	13	"
	160	—	7	0.44	13	"
	164	—	15	0.41	8	Moderately high
	166	—	15	0.45	7	" "
	198*	+	9	0.37	4	Normal
	192	+	14	0.22	7	Moderately high
	199*	+	23	0.71	7	" "
Moderate	194	+	8	0.39	9	" "
Dermatomyositis						
Far advanced	190	+	15	1.54	6	Moderately high
Early	193	+	6	0.18	4	Normal
Amyotonia Congenita						
Far advanced	196*	+	6	0.29	11	High
Myotonia Atropica						
Moderate	200*	0	3	0.08	3	Normal
Normal Humans	202	0	7	0.25	5	"
	213	0	5	0.18	1	"
	221	0	3.5	0.16	2	"
	223	0	5.3	0.28	6	"
	225	0	2.4	0.20	4	"

* Patients receiving therapy.

† Samples run after prolonged storage, + indicates creatine excretion; 0, no creatine; and —, no satisfactory determination.

‡ Two-dimensional chromatograms were secured only after several months storage at 0°C under thymol.

cases over 20. Any value over 10 indicates an increased urinary excretion of free amino acids. Normal urines were chromatographed and the amino-acid distribution was similar to that described by Dent.[4]

Two-dimensional partition chromatography was also employed, which resembles the strip method except that a second solvent is run at right angles to the first, giving a two-dimensional distribution of spots on a rectangular sheet of filter paper. We employed phenol saturated with water as the solvent for the first run, followed by 24-hour air drying and subsequent development in a 50-50 mixture of lutidine and collidine saturated with water. The sheets were then dried at room temperature, sprayed with ninhydrin and heated as before. The use of lutidine-collidine

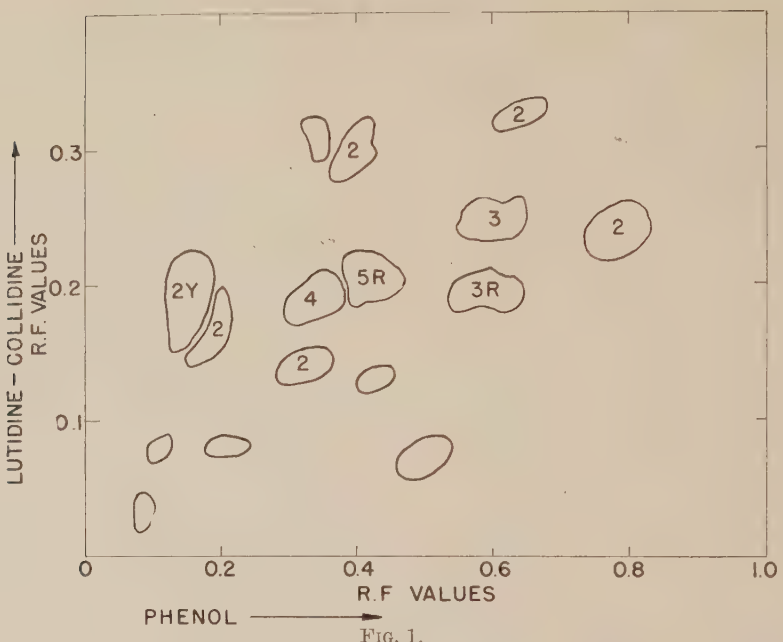

FIG. 1.

Two-dimensional Chromatogram of Urine in Progressive Muscular Dystrophy. The color intensity is indicated by numbers "1" to "5" (deepest color) as described in the text. Unnumbered spots have a color intensity of "1." The spots were purple with the exception of one yellow (Y) and two reddish (R) ones.

in preference to collidine alone was suggested by Dent.[6]

Our results with normal individuals correspond with those obtained by Dent.[4] Normal urine generally yields 2 or 3 spots in the two-dimensional technique and rarely more than 4. As shown in Table I, patients with progressive muscular dystrophy yield numerous spots indicating the excretion of a large number of amino acids. Eight amino acids represents an excretion which is moderately abnormal, and the appearance of 10 or more amino acids indicates a definitely pathological condition. In Fig. 1 is shown a diagram of a two-dimensional chromatogram of urine from a patient with progressive muscular dystrophy. The multiplicity of spots indicates an excessive excretion of amino acids.

In order to estimate the total amount of amino-acid excretion, the determination of amino nitrogen was performed on all urines by the method of Albanese and Irby.[7] This method depends upon the iodometric determination of copper obtained by filtration of the soluble amino acid copper salts. Albanese and Irby reported an amino nitrogen output of 200-700 mg per 24 hours in normal humans. This method yields high values when polypeptides are present and occasionally low values because certain of the amino acid copper salts are comparatively insoluble. As a result only partial correspondence was observed with the data obtained by partition chromatography. However, as may be seen in Table I, many of the values of amino nitrogen excretion are significantly higher than those observed in normal humans. An excretion of 0.50 mg amino nitrogen per ml or greater can be considered as an excessive excretion of amino nitrogen.

Urine of patients suspected of having muscular dystrophy is usually analyzed for creatine content. The finding of creatinuria would substantiate a clinical diagnosis of

[6] Dent, C. E., private communication.

[7] Albanese, A. A., and Irby, V., *J. Biol. Chem.*, 1944, **153**, 583.

dystrophy. In our work, we used a modified creatine determination using the technique of partition chromatography.[8] Those patients who excreted urines which do not show increased amino acid excretion likewise failed to show any significant creatine excretion as measured by this technique.

The amino nitrogen excretion normally represents only 2-4% of the total nitrogen excretion. It is evident that it would take an extremely large increase in amino nitrogen to raise significantly the value for total nitrogen as measured in the usual fashion. On the basis of the samples which are reported it would seem evident that these urines should be considered by all factors cited, that is, general description, *dw* value, number of spots, and milligrams of amino nitrogen excretion per ml. Comparison of the results of these various methods on urines from patients with progressive muscular dystrophy indicates that in many instances a generalized aminoaciduria is present. A general correspondence is observed between the data obtained by partition chromatography and that obtained by chemical amino nitrogen determination, but it is not close enough for the methods to replace one another. The number of amino acids excreted by a patient as indicated by the spots in two-dimensional paper chromatography is probably the most useful of the several determinations. It is possible for a patient to have a normal level of urinary amino nitrogen and still have a urine that would be characterized by an abnormally high excretion of certain amino acids. If the number of amino acids were large but the concentration of each were low, the total amino-nitrogen excretion would remain within normal limits. Details on the excretion of the individual amino acids in urines from humans with progressive muscular dystrophy will appear in a subsequent communication.

Summary. Two methods, partition chromatography and chemical amino nitrogen determination, have been employed in a study of the amino-acid excretions of 32 patients diagnosed as suffering from progressive muscular dystrophy and closely related conditions. In the case of progressive muscular dystrophy, partition chromatography indicated that both the number and the concentration of amino acids excreted were in many cases considerably above the normal limits. In addition, the results of amino nitrogen determinations indicated that some of these patients showed values far above normal limits and the majority were somewhat greater than normal.

Early muscular dystrophies, particularly of the facio-scapulo-humeral type, did not exhibit as marked an excretion of amino acids as the more advanced cases with or without pseudohypertrophic muscle symptoms. Data on the amino acid excretion of 2 cases of dermatomyositis and one each of amyotonia congenita and myotonia atropica were obtained.

On the basis of these observations it is concluded that progressive muscular dystrophy is characterized by a generalized aminoaciduria.

[8] Ames, S. R., and Risley, H. A., to be published.

16415

Isolation of Vitamin K_2 from Cultures of a Spore-Forming Soil Bacillus.

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During the course of an investigation of the bactericidal substances obtained from a spore-forming soil bacillus, *Bacillus brevis*, described by Dubos and Cattaneo,[1] we observed that ether extracts of the autolyzed bacilli gave a Dam-Karrer color reaction with alkali, characteristic of vitamin K.[2] When the oily residue obtained from the ether extracts was tested for antihemorrhagic activity in chicks[3] it was found to be fully effective at 100γ.

The K principle present in this residue was readily isolated by the method of Fieser for the isolation of vitamin K_1 from alfalfa concentrates.[4] Thus, according to this procedure 8 g of the above residue was suspended in 75 cc methanol to which was added 1 g of sodium hydrosulfite dissolved in 3 cc water. After shaking for 18 hours, the dihydrovitamin was taken into petroleum ether, washed with 2% aqueous potassium hydroxide containing sodium hydrosulfite until the washings were colorless, extracted with Claisen's alkali containing hydrosulfite; and recovered from the bright yellow liquor by dilution with water and extraction with petroleum ether. As the reduced form of this K factor is somewhat more soluble than dihydro-vitamin K_1,[4] the petroleum ether solution (2 cc) was chilled to −20°C, and the waxy solid which deposited was separated by centrifugation. After washing several times with petroleum ether by centrifugation at −20°C, the white, waxy hydroquinone dissolved in anhydrous ether was oxidized with silver oxide in the presence of anhydrous magnesium sulfate. On concentrating the ether solution, a bright yellow oil was obtained (80 mg) which crystallized after the addition of a few drops of cold acetone. By recrystallization from a mixture of chloroform and methanol the product was obtained as microscopic, yellow platelets which was identified as vitamin K_2 by a comparison of its properties with those already reported.[5] The product melted at 52.5 - 53.5°C (Found: C, 84.45; H, 9.99), and on reductive acetylation the dihydrodiacetate melting at 57 - 58°C was obtained (Found: C, 80.94; H, 9.86). In hexane, the ultraviolet absorption indicates sharp maxima at 243, 249 ($E^{1\%}_{1\ cm} = 520$), 260, 269 and a broad and less intense band at 310 to 340 with a maximum very close to 325. A comparative chick assay of vitamin K_2 with vitamin K_1 is shown in Table I. These results are in excellent agreement with those previously reported.[6]

Since ether extracts of the nutrient used for growing the organism (Bacto-Tryptone, Difco) were found to have some K activity, it was of interest to demonstrate whether the K_2 present in the incubated culture is actually

TABLE I.

Substance	Dose, μg	No. of chicks	Clotting time below 10 min.
K_1	1	9	8
K_2	1	9	6
	1.5	9	8
	2.0	9	9
Control	0	9	all 60 min.

[1] Dubos and Cattaneo, *J. Exp. Med.*, 1939, **70**, 249.

[2] Dam, Geiger, Glavind, P. Karrer, W. Karrer, Rothchild, and Solomon, *Helv.*, 1939, **22**, 310.

[3] Tishler and Sampson, *J.A.C.S.*, 1939, **61**, 2563.

[4] Fieser, *J.A.C.S.*, 1939, **61**, 2561.

[5] McKee, Binkley, MacCorquodale, Thayer, and Doisy, *J.A.C.S.*, 1939, **61**, 1295; Binkley, MacCorquodale, Cheney, Thayer, McKee, and Doisy, *J.A.C.S.*, 1939, **61**, 1612; McKee, Binkley, Thayer, MacCorquodale, and Doisy, *J. Biol. Chem.*, 1939, **131**, 327.

[6] Thayer, McKee, Binkley, MacCorquodale, Doisy, Proc. Soc. Exp. Biol. and Med., 1939, **41**, 194; Dam, Glavind, and Karrer, *Helv.*, 1940, **23**, 225.

produced by the organisms. Chick assays and quantitative analyses by the method of Trenner and Bacher[7] developed for vitamin K_1 proved that the yield of vitamin K_2 from the autolyzed bacilli is not significantly altered when the nutrient is previously extracted with ether. Furthermore biological assays on the nutrient itself indicated that not more than 10% of the vitamin present in the final culture can be introduced from this source.

The synthesis of a substance with K activity during putrefaction of fish meal and rice bran was first pointed out by Almquist and Stokstad.[8] It was found later that species of bacteria grown on the usual media also produced an antihemorrhagic factor.[9] No attempt, however, was made by these investigators to isolate and characterize the active principle. The well-known isolation of K_2 from putrefied fish meal[5] and this report of its isolation from the autolyzed cellular material of a spore-forming soil bacillus suggest that vitamin K_2 is a bacterial metabolite. In this connection the observation of Woolley and McCarter[10] that antihemorrhagic compounds as vitamin K concentrate, 2-methyl-1, 4-naphthoquinone, and phthiocol stimulates the growth of Johne's bacillus (*Mycobacterium paratuberculosis*) is noteworthy.

Summary A substance has been isolated from the autolyzed cellular material of the spore forming soil organism *Bacillus brevis* which, by both chemical and biological examination, is identical with vitamin K_2.

[7] Trenner and Bacher, *J. Biol. Chem.*, 1941, **137**, 745.

[8] Almquist and Stokstad, *J. Nutrition*, 1936, **12**, 329.

[9] Almquist, Pentler, and Meech, PROC. SOC. EXP. BIOL. AND MED. 1938, **38**, 336.

[10] Woolley and McCarter, PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 357.

16416

Effect of Amino Acid Deficiency on Rat Liver Before and After Partial Hepatectomy.*

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Since the remaining portion of rat liver after partial hepatectomy regenerates to normal size within 10 to 14 days it seemed of interest to see what effect a dietary deficiency of some essential amino acids might have on the formation of nucleic acids during this period of extremely rapid growth.

Partial hepatectomy was performed on rats that had been fed diets whose protein content was exclusively casein, zein, or gelatin. The livers at the time of hepatectomy and 4 days later were analyzed for ribonucleic acid, desoxyribonucleic acid, tryptophane, percentage regeneration, total nitrogen and total solids. Values for ribonucleic acid (P.N.A.) and desoxyribonucleic acid (D.N.A.) were obtained by the method of Schmidt and Thannhauser.[1] The determination of tryptophane was made by the procedure of Horn and Jones.[2] The percentage regeneration of the liver after partial hepatectomy is the increase in weight of the remnant as percentage of the amount of tissue removed. Since, on

* Aided by grants from the National Advisory Cancer Council Research Grants Division, U.S.P.H.S., and the Committee on Endocrinology, National Research Council.

[1] Schmidt, Gerhardt, and Thannhauser, S. J., *J.B.C.*, 1945, **161**, 83.

[2] Horn, M. J., and Jones, D. B., *J.B.C.*, 1945, **157**, 153.

the average, two-thirds of the liver is removed at partial hepatectomy,

$$\% \text{ Regeneration} = \frac{(\text{Wt of liver after regeneration} - \tfrac{1}{2}\text{wt of tissue removed})\ 100}{\text{Wt of tissue removed}}$$

Total solids were determined by drying the tissue to constant weight at 104°C. Micro-Kjeldahl with direct nesslerization was used for the nitrogen determinations. Differences were considered statistically significant when the Fischer t value exceeded 3.0.

The basic control diet consisted of 25% casein, 50% sucrose, 20% Crisco, 5% salt mixture (Phillips and Hart[3]) and a vitamin supplement of 0.5 ml "Vi Penta Drops" (Roche) per kilo. In the test diets the casein was replaced by zein or gelatin. One group of rats was given their maintenance ration of "Purina Chow" during the experimental period. The rats were kept on the respective diets for 10 days, then partially hepatectomized by the removal of the left lateral and median lobes. The rats continued on their diets during the 96 hour period of regeneration following the hepatectomy and then were killed for the final analysis of the livers. The 96 hour period was chosen because it has been shown that the most active cellular proliferation occurs within 72 to 96 hours.[4,5]

Results. (1) *Pre-Hepatectomized Liver.* The total solids and both P.N.A. and D.N.A. were the same on the zein and gelatin diets as on casein and Purina, while the tryptophane, though lower, was not greatly so. The total nitrogen on gelatin was also somewhat lower, though the significance was questionable, but was markedly reduced on the zein as compared with the other diets. Kosterlitz and Campbell[6] observed that in rats on a diet of zein with added lysine or tryptophane there was a considerable loss of liver protein as measured by non-lipid, non-glycogen liver solids. In a later paper Kosterlitz[7] reported a similar finding with an 18% gelatin diet, but he observed no change in total nucleic acid concentration of the liver. However, when his results were calculated per 100 g initial body weight instead of per 100 g liver weight, decreases in protein, phospholipin and nucleic acid were found. From indirect evidence he suggests that only cytoplasmic P.N.A. and not nuclear D.N.A. is affected. Later, by direct determination, Campbell and Kosterlitz[8] found that the D.N.A. content of liver tissue of rats, calculated for 100 g of initial body weight was not influenced by the protein content of the diet while P.N.A. was lost as had been previously suggested.

(2) *Regenerating Liver.* The regenerating liver, after 96 hours, has, we find, the same content of total solids on all diets, and is unchanged from the pre-hepatectomy liver. The concentration of D.N.A. in the regenerating livers of rats on the casein and purina diets was the same as that of the organ before hepatectomy. The P.N.A. content, however, was significantly greater. This differs from the observations of Davidson and Waymouth[4] who found no significant difference between the nucleoprotein of livers before and after partial hepatectomy. This extra mobilization of P.N.A. was observed also on a gelatin diet, but not when zein was the sole source of protein. The D.N.A. on these two diets was not significantly altered after hepatectomy.

Our values for tryptophane in the regenerating livers of rats on all diets tended to be lower than that of liver before hepatectomy, but not significantly so. In regenerating liver, the tryptophane values were somewhat less on the zein and gelatin diets than on the control casein diets, yet the striking thing was that they were so nearly alike, in spite of the fact that tryptophane was lacking in 2 of the diets.

Brues, Drury, and Brues[9] reported that the nitrogen content of regenerating liver after partial hepatectomy rose from a low value of 2.67% on the first day, but was still below normal (3.51%) well into the second week.

[3] Phillips, P. H., and Hart, E. B., *J.B.C.*, 1935, **109**, 657.

[4] Davidson, J. N., and Waymouth, C., *Biochem. J.*, 1944, **38**, 385.

[5] Drabkin, D. L., *J.B.C.*, 1947, **171**, 395.

[6] Kosterlitz, H. W., and Campbell, Rosa M., *J. Physiol.*, 1945-46, **104**, 16P.

[7] Kosterlitz, H. W., *J. Physiol.*, 1947, **106**, 194.

[8] Campbell, R. M., and Kosterlitz, H. W., *J. Physiol.*, 1947, **106**, 12P.

TABLE I.
Analyses of Livers.

Diet	Total solids, %	Nucleic acid phosphorus mg/100 g wet wt		Tryptophane, % wet wt	Total N, % wet wt	% Regeneration
		P.N.A.	D.N.A.			
		At hepatectomy.				
Purina	31 ± 0.1* (9)	99 ± 2.3 (25)	23 ± 1.1 (25)		3.45 ± 0.07 (9)	
Casein	29 ± 0.2 (18)	99 ± 2.8 (15)	23 ± 1.1 (20)	0.25 ± 0.01 (15)	3.37 ± 0.08 (13)	
Zein	30 ± 1.0 (26)	99 ± 1.7 (13)	23 ± 3.0 (13)	0.22 ± 0.01 (11)	2.49 ± 0.14 (11)	
Gelatin	28 ± 1.0 (21)	96 ± 1.7 (19)	27 ± 1.7 (19)	.021 ± 0.007 (19)	3.07 ± 0.08 (15)	
		After 96 hr regeneration.				
Purina	27 ± 0.5 (10)	111 ± 1.5 (10)	20 ± 2.2 (9)		2.97 ± 0.07 (10)	61 ± 6.7 (10)
Casein	30 ± 1.1 (15)	115 ± 3.4 (16)	25 ± 2.0 (16)	0.22 ± 0.005 (14)	3.17 ± 0.12 (11)	67 ± 3.5 (16)
Zein	29 ± 0.8 (14)	98 ± 2.2 (15)	19 ± 1.2 (14)	0.21 ± 0.012 (10)	2.36 ± 0.11 (10)	63 ± 2.6 (23)
Gelatin	28 ± 1.0 (15)	109 ± 3.0 (17)	20 ± 1.4 (17)	0.19 ± 0.007 (13)	2.94 ± 0.14 (11)	73 ± 4.5 (15)

* Standard deviation of the mean.
Figures in parentheses indicate the number of animals.

In our experiments, however, the nitrogen concentration of the regenerated liver of rats on the casein diet, though somewhat lower than before hepatectomy, was not significantly so except on a "Purina Chow" diet. On the zein diet (but not on gelatin) we did observe a marked lowering of the nitrogen concentration in regenerating liver as compared with the casein control, the t value for this difference being 5.0. This is in line with the findings of Harrison and Long[10] who reported that zein, gelatin and gliadin were inadequate for the restoration of liver protein following depletion by a 48 hour fast. They used liver nitrogen in mg per 100 g of body weight as a measure of cytoplasmic protein.

Conclusion. 1. The total weight and total solids of regenerated liver 96 hours after partial hepatectomy are unaffected by diets in which gelatin and zein are the sole sources of protein.

2. On a casein diet, the regenerated liver has a higher P.N.A. content than the pre-hepatectomy liver. This is also true on a gelatin diet and on a purina chow diet, but not on a zein diet, indicating that the apparent stimulus to extra P.N.A. content normally induced in the regenerating liver, is inhibited by the lack of dietary lysine. There is no significant effect on the D.N.A. content either before or after hepatectomy, on any of the diets.

3. On gelatin and zein diets, the tryptophane content of the pre-hepatectomy livers was only slightly less than on adequate diets; the same held true for the regenerating liver, indicating that the liver was capable of obtaining almost its normal content of needed tryptophane from other sources.

4. In contrast to the constancy of the total solids, the total nitrogen of both the pre-hepatectomy and regenerating liver was significantly less on the zein diet than on the Purina chow, casein, and gelatin diets. This implies that on the zein diet, the decreases in nitrogen were associated with an increase in other constituents. On the basis of Kosterlitz's (loc. cit.) results, this may have been glycogen.

We are indebted to Corn Products Refining Company for the supply of zein.

[9] Brues, A. M., Drury, D. D., and Brues, M., *Arch. Path.*, 1936, **22**, 658.

[10] Harrison, Helen C., and Long, C. N. H., *J.B.C.*, 1945, **161**, 545.

16417

Demethylation of Carcinogenic Aminoazo Dyes by Autoxidizing Linoleic Acid.*

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Previous publications from this laboratory have demonstrated that various carcinogens including certain azo dyes inhibit the autoxidation of unsaturated lipids.[1,2] Destruction of the carcinogenic hydrocarbons was shown to occur during the course of the oxidation[3,4] and there were indications that the carcinogenic aminoazo dyes likewise disappeared in the presence of autoxidizing lipids. The present paper describes in more detail the effect of p-dimethylaminoazobenzene and related compounds on the autoxidation of linoleic acid and provides some information on the fate of these dyes during the oxidation.

Methods. Most of the work was done with p-dimethylaminoazobenzene (DAB), p-monomethylaminoazobenzene (MAB), and p-aminoazobenzene (AB); m′-methyl-p-dimethylaminoazobenzene, p′-methyl-p-dimethylaminoazobenzene, p-ethylmethylaminoazobenzene, and p-diethylaminoazobenzene were also tested for their antioxidant activity. These dyes were prepared as described previously[5] and purified by chromatographic adsorption on alumina.[6] We are indebted to Dr. J. B. Brown of the Department of Physiological Chemistry at Ohio State University for the linoleic acid. This preparation was a highly purified product (I.N. = 180.7) obtained by fractional freezing. In general 9.025 mg of linoleic acid and varying levels of dye to give M/200 to M/50 solutions were placed in Warburg flasks and the rate of autoxidation followed manometrically at 37.5°. The various constituents were added to the flasks in petroleum ether and the solvent was then thoroughly removed *in vacuo* at room temperature. The mixture of fatty acid and dye remained on the bottom of the vessels in the form of small droplets. In the studies with DAB, MAB, and AB flasks were removed during the course of the autoxidation and the contents transferred by two washings with 2 ml portions of each of the following solvents in the order named: 11 N KOH, water, and 95% ethanol. Quantitative determinations of the mixtures for DAB, MAB, and AB were done according to the chromatographic method of Miller and Baumann.[6]

Results. Fig. 1 shows the effect of DAB, MAB, AB on the autoxidation of linoleic acid (LA) at a concentration of dye in acid of M/100. DAB and MAB both increased the latent period of autoxidation of linoleic acid, the former being a more effective antioxidant than the latter. The antioxidant effect of each dye was proportional to the concentration employed. Thus, when DAB was used at concentrations of M/200, M/100, and M/50 the oxidation of the linoleic acid at the end of the first 24 hour period had reached only 56, 29, and 0% respectively that of the fatty acid alone. With the same levels of MAB, the amount of oxidation was 73, 45, and 35%

* Aided by grants from the United States Public Health Service on recommendation of the National Advisory Cancer Council and the Jane Coffin Childs Memorial Fund for Medical Research.

1 Rusch, H. P., and Kline, B. E., *Cancer Research*, 1941, **1**, 465.

2 Deutsch, H. F., Kline, B. E., and Rusch, H. P., *J. Biol. Chem.*, 1941, **141**, 529.

3 Mueller, G. C., Miller, J. A., and Rusch, H. P., *Cancer Research*, 1945, **5**, 401.

4 Mueller, G. C., and Rusch, H. P., *Cancer Research*, 1945, **5**, 480.

5 Miller, J. A., and Miller, E. C., *J. Exp. Med.*, 1948, **87**, 139.

6 Miller, J. A., and Baumann, C. A., *Cancer Research*, 1945, **5**, 157.

respectively. Repetition of the experiments revealed excellent agreement in the results. Other dyes with approximately the same inhibiting effect as DAB were m′-methyl-p-dimethylaminoazobenzene, p′-methyl-p-dimethylaminoazobenzene, and p-ethylmethylaminoazobenzene. In contrast to the inhibitory effect of the methylated dyes, p-diethylaminoazobenzene had little effect and AB did not alter the latent period. In several experiments, AB actually caused a slight early stimulation of oxidation. However, the total oxygen uptake was decreased with the non-methylated dyes almost as much as it was for the methylated dyes; the chief difference was noted in the length of the latent period.

During the latent period an output of gas was observed with linoleic acid when mixed with all but one of the azo dyes. The exception was AB (Fig. 1). The gas evolved was probably carbon dioxide since the effect could be overcome by the addition of KOH to the center wells of the Warburg flasks. It is of interest that a considerable amount of gas absorbable by KOH was also evolved by the autoxidation of linoleic acid alone; after 96 hours of autoxidation about 200 microliters was evolved from 9.025 mg of linoleic acid. This was only observed toward the end of the period of oxidation, since the total uptake of gas was greater and the plateau was reached later when alkali was present in the wells of the flasks.

As the autoxidation proceeded in the acid-dye mixtures the azo dyes disappeared and DAB and MAB were found to be demethylated during the course of their destruction. At the end of 30 hours 90% of the DAB initially added had disappeared but MAB appeared in amounts equal to 85% of the starting level of DAB; almost a quantitative conversion had occurred up to this point (Fig. 2). Thereafter, the amount of MAB gradually decreased and small amounts of AB were also found throughout the period of oxidation. MAB was likewise destroyed when added to linoleic acid, but the rate of disappearance was slower than that with DAB; again demethylation to form AB occurred (Fig. 3). Added AB disappeared very rapidly in autoxidizing linoleic acid and no other basic dye was detected in the mixture (Fig. 4). Demethylation occurred only when the linoleic acid was oxidizing, and no disappearance of dye was observed when the autoxidation was retarded by tocopherol. There was no evidence to indicate that the demethylation

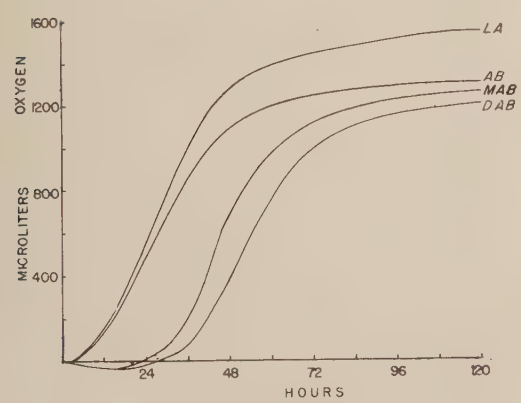

FIG. 1.

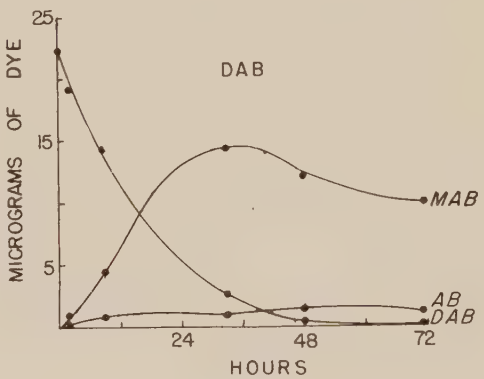

FIG. 2.

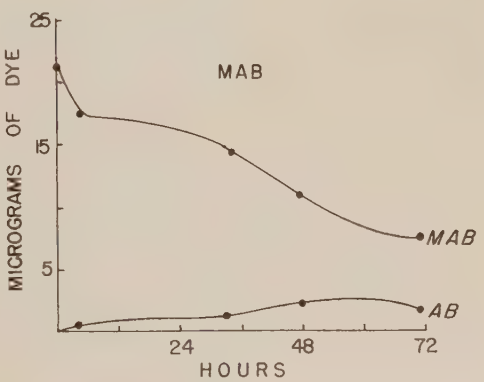

FIG. 3.

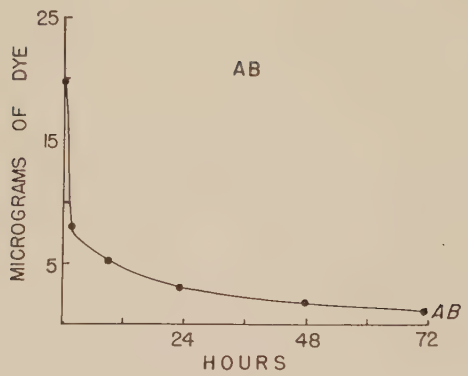

FIG. 4.

was a reversible reaction, since no DAB was detected during the autoxidation of either MAB or AB or a combination of the two.

Discussion. The product of oxidation of the methyl groups removed from the amino nitrogen of these dyes is unknown. However, the fact that they are replaced by hydrogen indicates that oxidation to easily decarboxylated carbamic acids probably occurred. It was not possible to demonstrate the oxidation of the methyl groups to carbon dioxide in this system since even the complete oxidation of the whole dye molecule could account for only a small part of the carbon dioxide evolution that occurred during these autoxidations. Although DAB is stable in the various diets that we have used in studies on tumor induction,[7] Kensler and his associates[8] have found that demethylation of DAB occurs *in vitro* when the dye is dissolved in cottonseed oil and mixed with ground brown rice. Heating the rice largely prevented the destruction of the dye. It seems likely that a coupled oxidation similar to that described in this paper occurred in the rice-cottonseed oil mixture. The loss of methyl groups on the oxidation of other dimethylamino compounds *in vitro* is implicit in the older literature. For example, Bernthsen[9] and Kehrmann[10] obtained demethylated products from the oxidation of methylene blue.

It is interesting that DAB suffers a similar fate *in vivo*. In the rat this dye is reversibly demethylated to form MAB which is then irreversibly demethylated to form AB.[11] The role of oxidizing lipids in the demethylation of this dye *in vivo* has not been ascertained.

Only rough correlations can be made between the antioxidant effect of these dyes and their carcinogenic activity.[5] Thus DAB and MAB are of equal carcinogenic activity but DAB retards oxidation more effectively than MAB. Similarly AB and p-diethylaminoazobenzene each lack carcinogenic and antioxidant capacity. On the other hand m′-methyl-p-dimethylaminoazobenzene, p′-methyl-p-dimethylaminoazobenzene, and p-ethylmethylaminoazobenzene are antioxidants of approximately equal activity. Yet the first of these three dyes is more active as a hepatic carcinogen than DAB, and the last compound is equal in its carcinogenic activity to that of DAB, whereas the p′-methyl-derivative is a very weak carcinogen.

Summary. p-Dimethylaminoazobenzene and p-monomethylaminoazobenzene both increased the latent period of autoxidation of linoleic acid, the former being a more effective antioxidant than the latter. The antioxidant effect of each dye was proportional to the concentration employed. Other dyes with approximately the same inhibiting effect as p-dimethylaminoazobenzene were m′-methyl-p-dimethylaminoazobenzene, p′-methyl-p-dimethylaminoazobenzene, and p-ethylmethylaminoazobenzene. In contrast p-diethylaminoazobenzene had only a slight effect and p-aminoazobenzene had no inhibitory influence. During the latent period an output of gas was observed when any of the methylated dyes were mixed with the linoleic acid; a gas output was also found with autoxidizing linoleic acid alone at the end of the oxidation period. The gas evolved was probably carbon dioxide.

As autoxidation of the linoleic acid-dye mixtures proceeded demethylation of p-dimethylaminoazobenzene and p-monomethyl-

[7] Miller, J. A., Kline, B. E., Rusch, H. P., and Baumann, C. A., *Cancer Research*, 1944, **4**, 158.

[8] Kensler, C. J., Magill, J. W., and Sugiura, K., *Cancer Research*, 1947, **7**, 95.

[9] Bernthsen, A., *Ber. Deut. Chem. Ges.*, 1906, **39**, 1804.

[10] Kehrmann, F., *Ber. Deut. Chem. Ges.*, 1906, **39**, 1403.

[11] Miller, J. A., Miller, E. C., and Baumann, C. A., *Cancer Research*, 1945, **5**, 162.

aminoazobenzene occurred. At the end of 30 hours, 90% of the p-dimethylaminoazobenzene initially added had disappeared and as much as 85% was accounted for in the form of the monomethyl derivative. Thereafter, the amount of p-monomethylaminoazobenzene decreased. Small amounts of p-aminoazobenzene also were formed. The demethylation occurred only during autoxidation; none was found when the oxidation was inhibited with tocopherol.

16418

Ergotoxine Hyper- and Hypothermia in Albino Rats.*

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In 1906 an alkaloid was isolated from ergot of rye by Barger and Carr[1] and called ergotoxine. A year later Barger and Dale[2] reported that "the high temperature at and after death seemed to be a characteristic effect of fatal doses of ergotoxine." Since these fundamental studies and throughout the succeeding 40 years, other alkaloids have been isolated and identified until at present we have to deal with 6 natural alkaloids of different composition, each with 2 isomers. Stoll and Hofmann[3] have shown that the compound called ergotoxine is not a uniform chemical substance, but a molecular complex of 3 alkaloids: ergocristine, ergocornine, and ergokryptine.

Sollmann,[4] in his discussion of the central nervous system effects of ergotoxine as shown by several investigators, stated that the body temperatures of rabbits are raised from 3 to 4.5° C by the intravenous injection of .5 to 1.5 mg per kg of ergotoxine. Since there was no rise in decapitated animals, it was assumed that the action must be central, and that it probably involved both increased heat production and diminished heat loss.

Goodman and Gilman,[5] on the other hand, considered the action of ergotoxine on the central nervous system, when administered in moderate amounts, to be variable and, as a rule, unimportant. They noted that large doses of ergotoxine, given intravenously in certain laboratory animals, produced marked excitement, sham rage, and "other evidence of stimulation of central sympathetic centers."

Githens[6] found that cats responded to ergotoxine by a rise of temperature, but that rats, mice and pigs exhibited falls in temperature. Youmans and Trimble[7] reported that ergotoxine produced practically no effect on body temperature in dogs. Rothlin,[8] in discussing the effect of the ergot alkaloids on temperature regulation, maintained that the action was of a central nature because it was suppressed by general anesthesia. Ergotamine, which according to Sollmann,[4] and Goodman and Gilman,[5] gives essentially the

* Supported by a grant from the Office of Naval Research.

1 Barger, G., and Carr, F. H., *J. Chem. Soc.* (London), 1907, **91**, 337.

2 Barger, G., and Dale, H. H., *Biochem. J.*, 1907, **2**, 240.

3 Stoll, A., and Hofmann, A., *Helv. chim. Acta.*, 1943, **26**, 1570.

4 Sollmann, T., *A Manual of Pharmacology*, 7th ed., W. B. Saunders Co., Philadelphia, 1948. 403.

5 Goodman, L., and Gilman, A., *The Pharmacological Basis of Therapeutics*, The Macmillan Co., New York, 1941, 658.

6 Githens, T. S., *J. Pharm. and Exp. Therap.*, 1917, **10**, 327.

7 Youmans, J. B., and Trimble, W. H., *J. Pharm. and Exp. Therap.*, 1930, **39**, 201.

8 Rothlin, E., *Bull. de l'Acad. Suisse des Sci. Med.*, 1946-47, **2**, 249.

same pharmacologic effects as ergotoxine, also raised the body temperature when given in toxic doses by Rothlin. Small doses of ergotamine, on the other hand, resulted in a depression of body temperature. Rothlin's observations were made on the rabbit and also indicated that ergocristine and the other alkaloids of ergotoxine do not lower, but rather produce elevations of, body temperature.

Sawyer and Schlossberg[9] studied the effects upon the body temperatures of ergotamine-treated cats of exposure to warm and cold environments. They injected 0.5 mg per kg of ergotamine tartrate intramuscularly and found that their animals were then unable to regulate their temperatures as well in abnormal environments as were normal cats. The deleterious effect in the warm environment was regularly more pronounced than that observed in the cold.

Methods and Materials. Ergotoxine ethanesulfonate† has been used in our investigations of temperature regulation in albino rats. Ergotoxine was originally selected as a means of investigating this mechanism because of its having been reported to have a thermogenic effect when administered to certain animals and because its thermogenicity has been accounted for on the basis of central (hypothalamic) stimulation. It has, in fact, proven to be a satisfactory thermogenic agent in the white rat in spite of previous reports to the contrary (Githens[6]).

After considerable experimentation it was found that the most satisfactory solvent for the ergotoxine ethanesulfonate was dilute ethyl alcohol, prepared by diluting ¼ cc of 50% alcohol to 2 cc with distilled water. One mg of the drug was dissolved in this amount of solution. The dilute alcoholic solution, by itself, when injected intraperitoneally, failed to produce any effect upon body temperature. Although never used routinely, saline suspensions (1 mg ergotoxine in 2 cc physiological saline) and a solution of 1 mg of the drug in 2 cc of 1/1000 normal hydrochloric acid were found to be as effective in producing ergotoxine fever in our rats as the dilute alcoholic solution.

The dose of ergotoxine utilized in the experiments reported herein has been 4.5 mg per kg and this has been administered intraperitoneally. This dosage can be given without toxic effects although rats receiving it show considerable increase in respiratory rate and a rather general increase in skeletal muscular activity. Even when this dosage is combined with long periods of exposure to cold (5-8° C) the animals recover completely, and will survive several subsequent administrations of the drug, combined with cold stress.

As quickly as possible, after injection of ergotoxine, records of the body temperatures of the experimental animals and of suitable controls were started. The control animal, in each experiment, was simultaneously subjected to all the procedures, including cold stress, to which the experimental animal was subjected, except for the administration of ergotoxine. Temperatures were recorded from each of the animals at one-minute intervals by means of a 2-channel Brown electronic recording potentiometer and copper constantan thermopiles inserted into the colon to the level of the diaphragm as previously described.[10]

Four main types of experiments have been carried out to determine: (1) the effects of ergotoxine upon rats whose environmental temperatures remain within the normal range of 28 to 31° C; (2) the effects of transferring ergotoxine-treated animals to a cold environment (5-8° C) after their hyperthermias have run their course; (3) the effects of exposure to cold at the height of the ergotoxine fever; and (4) the effects of exposure to cold immediately after administration of ergotoxine.

The effects of subcutaneous injections of ergotoxine have also been observed and com-

[9] Sawyer, M. E. M., and Schlossberg, T., *Am. J. Physiol.*, 1933, **104**, 172.

† The ergotoxine ethanesulfonate was very kindly furnished by the Wellcome Research Laboratories, Tuckahoe, N. Y.

[10] Buchanan, A. R., and Hill, R. M., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 602.

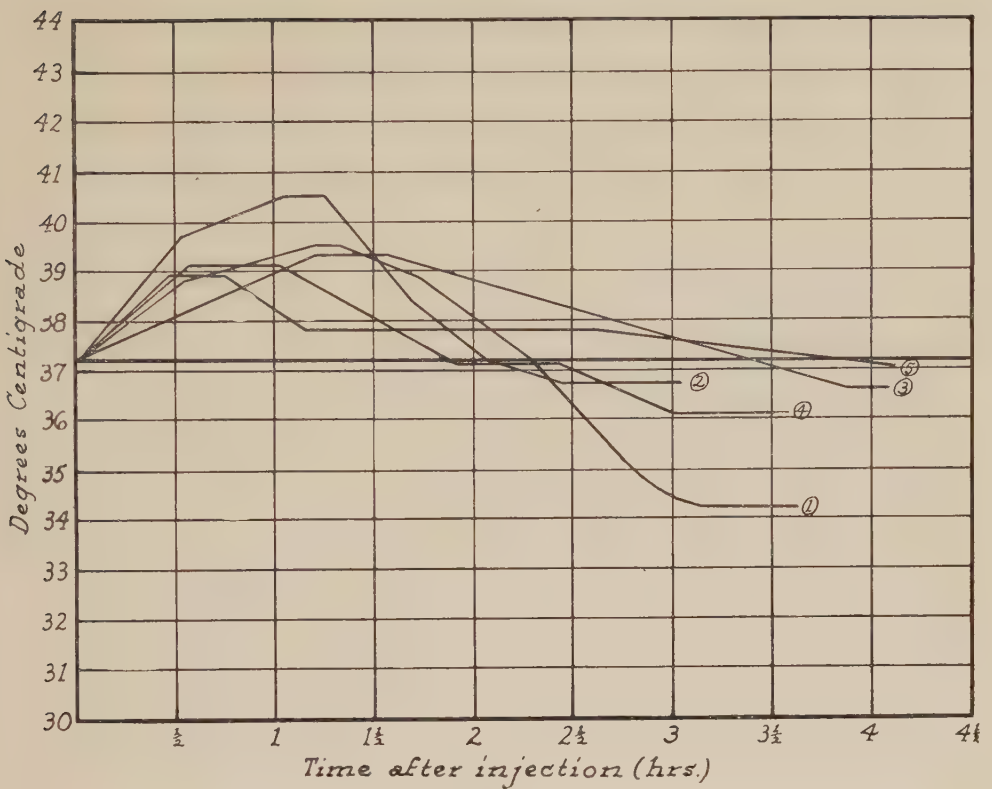

FIG. 1.
Graphic representation of the hyperthermic reactions recorded from 5 ergotoxine-treated rats in a 28-31° C environment.

pared with those produced by intraperitoneal administration. Ergotoxine (4.5 mg per kg) and urethane (0.25 g per kg) have been administered simultaneously in order to determine whether the typical ergotoxine fever is suppressed by general anesthesia. Mild heat stress has been applied to a considerable number of our ergotoxine-treated rats in order to learn whether the drug also interferes with the ability to regulate against heat.

Finally, the effects upon the internal temperatures of ergotoxine-treated rats of minimal changes in their external environmental temperatures have been investigated by utilizing a small incubator placed in the cold room. This arrangement has enabled us to produce at will any environmental temperature between that of the cold room (5-8° C) and that which is considered normal for our animals (28-31° C).

Wistar Strain rats from our own colony, as well as Denver University Strain rats, have been utilized in this study and the same results have been obtained in both. All have been regulating animals[10] and practically all have been fully developed adult rats.

Results. Intraperitoneal injections of ergotoxine ethanesulfonate in doses of 4.5 mg per kg have consistently resulted in significant rises in body temperatures of albino rats when the environmental temperature ranged between 28 and 31° C. The total hyperthermic reaction of a given animal, in degree-minutes, may be calculated from the total area under the graph in a continuous temperature record from the time of administration of the drug to the time when the temperature returned to its initial level. An environmental temperature of 31° was conducive to the development of more fever than one of 28°. If the experiment was continued in the same environment, beyond the period of ergotoxine hyperthermia,

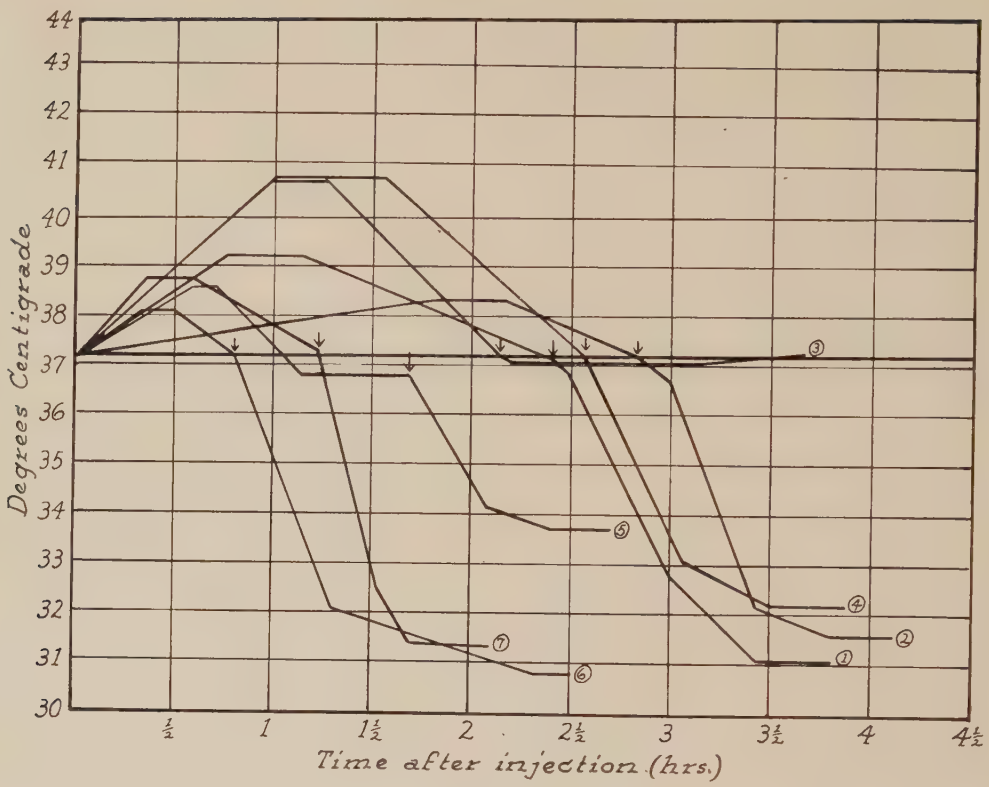

FIG. 2.

Graphic representation of hyperthermic reactions in 7 ergotoxine-treated rats and of hypothermia in the same animals as the result of exposure to the cold environment (5-8°C). The time at which each rat was exposed is indicated by an arrow.

the temperature of the rat fell below its initial (pre-hyperthermic) level.

The average hyperthermic reaction in 14 ergotoxine-treated rats, 12 of which are included in Fig. 1 and 2, amounted to 122 degree-minutes with a range from 26 to 283. The average maximum reading was 2° C above the initial temperature with a range from 1.1° to 3.6° and the average rate of rise was 3.1° C per hour (Fig. 1 and 2).

Placing ergotoxine-treated rats in the cold room (5-8° C) when their temperatures reached pre-hyperthermic levels resulted in rapid temperature falls (Fig. 2). These continued to rather low levels and their average rate was 9.2° per hour with a range from 6° to 15.6°.

Examination of Fig. 1, 2, 3, and 4 reveals that the time-temperature graphs obtained from our experimental rats consist essentially of linear segments with sharp inflection points. Curved segments have been indicated only where 10% of the points in the original record deviated 0.2° or more from a straight line. The temperature records have been simplified and their visualization made easier by shifting all of them as necessary to bring the initial temperature of all animals to a standard figure of 37.2° C.

Those rats transferred to the cold environment at the height of their fever (Fig. 3) have shown immediate falls in temperature which have proceeded at an average rate somewhat faster than that observed in animals placed in the cold environment at the termination of hyperthermia (10.2° C per hour).

The administration of ergotoxine in the same dosage to rats which are immediately exposed to the cold environment does not usually produce an increase in body tempera-

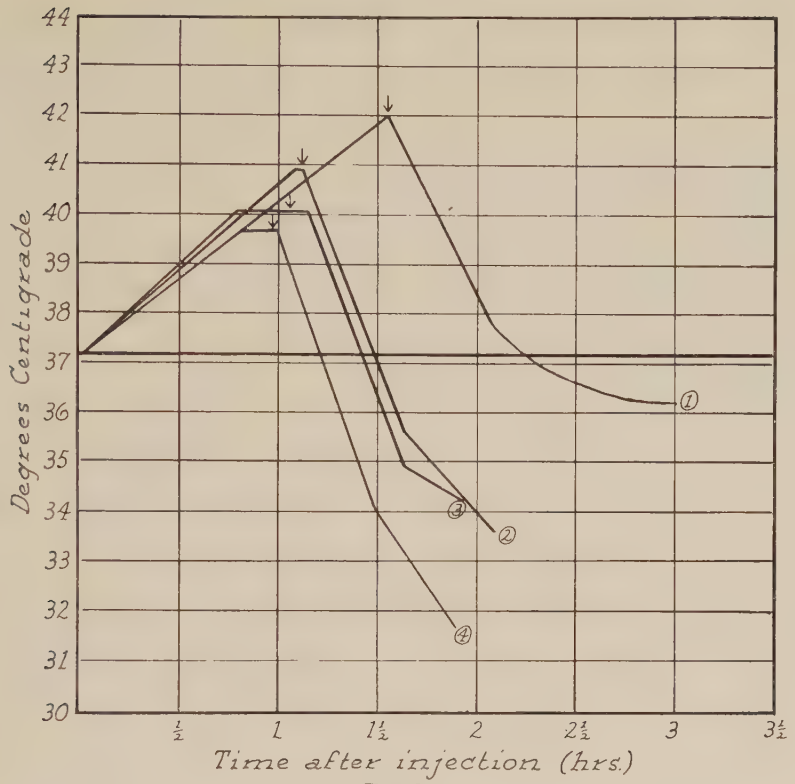

FIG. 3.

Temperature falls in 4 rats exposed in the cold room at the heights of their fevers. The times at which exposures began are indicated by arrows.

ture (Fig. 4). If the animals are placed in the cold environment (5-8° C) quickly enough after administration of the drug, there is no increase in temperature, but if there is a slight delay, a transient increase may appear; this is followed by a marked fall. For practical purposes, it may be stated that ergotoxine in the dosage which produces hyperthermia in an environmental temperature of 28-31° C results in a marked fall in body temperature when the animals are immediately exposed to cold. The average rate of fall in such animals is 7.1° C per hour, with a range from 3.9° to 9°.

Subcutaneous administration of ergotoxine to white rats in the same dosage and in the same medium described above, has not been sufficiently investigated for definite conclusions to be drawn. With the animal in a 28-31° C environment, it has so far always produced hyperthermia which is slower to appear, longer-lasting, and not as high as that produced by intraperitoneal injection.

Urethane (0.25 g per kg) has been given to a rat which had previously reacted to ergotoxine with a typical fever. The urethane, as such, had no effect upon body temperature. When, subsequently, the same animal received the same quantity of urethane together with the usual dose of ergotoxine, it failed to manifest any hyperthermia at an environmental temperature of 28-31° C; exposure to cold, however, resulted in a marked fall in temperature which was comparable to that seen in ergotoxine-treated animals without anesthesia. Subsequent injections of ergotoxine in other animals, and without urethane, have always produced hyperthermic reactions as marked as, or more marked than, that produced by the original injection.

Instability of regulation against mild heat stress has regularly been demonstrated in

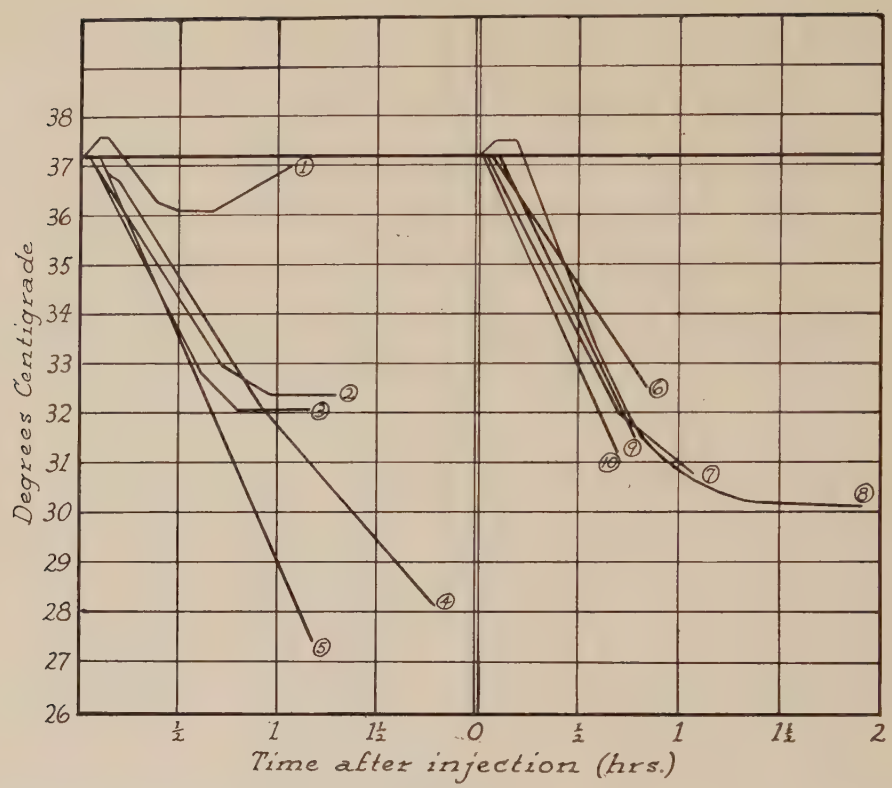

FIG. 4.

Temperature falls in 10 rats which were exposed to cold immediately after administration of ergotoxine. The animals have been separated into two groups only to prevent the confusion which would result from attempting to include all of them in a single graph.

animals given ergotoxine. It must be noted, however, that normal rats regulate poorly against heat and that the instability demonstrated in ergotoxine-treated animals is relative rather than absolute.

The marked sensitivity of experimental rats to changes in the temperature of their external environment has been particularly well demonstrated in those animals observed while in the incubator which, in turn, was operating in the cold room. It has been repeatedly shown, in such experiments, that the body temperatures of ergotoxine-treated rats placed in and maintained at incubator temperatures of 22-25° C may either rise or fall initially, after which they remain very constant within a rather narrow range. If the thermostat of the incubator is adjusted downward to such an extent that, after a considerable period of slow cooling, the new environmental temperature stabilizes at a level 3 degrees below its original level, the rat's internal temperature falls almost as rapidly as that of its environment and almost as much (2.5° C).

Discussion. Two possible explanations may be proposed as to why Githens[6] observed falls in the temperatures of white rats after administration of ergotoxine as contrasted to the rises which were consistently observed by us. The first of these relates to the difference that has been noted between rectal and high colonic temperatures. Hill *et all.*[11] have shown that this difference averages 3.5° C and that rectal temperatures are much more subject to modification by the external environment. They are probably also modified by the peripheral vasoconstriction said to result from the direct action of ergotoxine on

[11] Hill, R. M., Ware, A. G., and Schultz, F. H., *Cancer Research*, 1943, **3**, 839.

the smooth musculature of peripheral vessels. Githens' temperatures were rectal temperatures while ours were all obtained from the colon at or near the level of the diaphragm.

A second way in which the discrepancy between our results and those of Githens may be explained suggests itself in the light of our observations as to the extreme sensitivity of ergotoxine-treated rats to their environmental temperatures. Hyperthermia was a consistent result of ergotoxine administration to our rats in an environmental temperature of 28-31° C. When the drug was given to rats which were immediately placed in a 22-25° C environment, hyperthermia was absent or of slight degree and of short duration; in some animals there was an immediate fall. Githens may have conducted his experiments in a considerably cooler environment than that in which the majority of ours were conducted or may possibly have overlooked an early and transient rise in temperature, since his first reading was made 30 minutes after injection of ergotoxine.

Increases in temperature resulting from the administration of ergotoxine and ergometrine to albino rabbits were studied by de Beer and Tullar[12] who also analyzed the sources of error attributable to animal variation and environmental influences. With regard to the latter, they recognized that room temperature may alter the hyperthermal response, an observation which, as previously mentioned, was also made by Sawyer and Schlossberg.[9] The latter investigators, however, reported temperatures obtained with a clinical thermometer from the groin; such temperatures would certainly be affected by the peripheral vascular reactions ordinarily attributed to ergotoxine, by those due to the environmental temperature itself, and by a combination of both.

It is interesting to speculate as to whether the thermal changes observed by us and by previous investigators are due to direct action upon the hypothalamus and, if so, whether the effect is due to stimulation or depression of the heat-regulating centers therein. At the present time we are inclined toward the opinion that ergotoxine stimulates the hypothalamus directly and, so far as its heat-regulating centers are concerned, unselectively. Whether this stimulation is manifested by hyper- or hypothermia apparently depends to considerable degree upon the environmental temperature. It appears reasonable to assume, further, that our "normal" environment (28-31° C) provides just sufficient advantage for the heat-production and heat-conservation centers that the effects of their stimulation overbalance those of stimulation of the heat-loss center. A cool or cold environment, conversely, provides an advantage in favor of the heat-loss center and hypothermia results.

It is admitted that the above concept of the mechanisms responsible for the phenomena observed and reported herein, ignores the direct vasoconstrictor effect on peripheral vessels usually attributed to ergotoxine. One might, perhaps, assume that peripheral vasoconstriction occurs in the "normal" environment and thus contributes to hyperthermia while the "sympathetic reversal" of Dale,[13] due to increased secretion of adrenaline, may occur in cooler environments and result in peripheral vasodilatation; this reaction may be particularly pronounced when the animals are abruptly exposed to the cold environment (5-8° C).

Summary. Intraperitoneal administration of ergotoxine to albino rats has consistently resulted in hyperthermic reactions when the environmental temperature has been 28° C or higher. When ergotoxine-treated rats were placed in an environment whose temperature varied between 5° and 8° C, hypothermia always resulted; this was true of immediate exposure and of exposure subsequent to and during hyperthermia. Environmental temperatures between 22° and 25° C were sometimes conducive to moderate rises in temperature and sometimes to moderate falls; these changes were of short duration and were followed by long periods in which the body temperature remained within a very narrow range. Urethane given in conjunction with

[12] de Beer, E. J., and Tullar, P. E., *J. Pharm. and Exp. Therap.*, 1941, **71**, 256.

[13] Dale, H. H., *J. Physiol.*, 1913, **46**, 291.

ergotoxine eliminated the hyperthermic effect. Subcutaneous administration of ergotoxine gave rise to less dramatic hyperthermic responses and to hypothermia (in cold environments) that was essentially the same as that elicted by intraperitoneal injection.

The authors wish to thank Dr. R. W. Whitehead, Professor of Physiology and Pharmacology, University of Colorado Medical School, for valuable suggestions and criticisms.

16419

The Changes in the Electrocardiogram Associated with Standing.*

DAVID SCHERF AND MILTON SCHLACHMAN.

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Differences of opinions concerning the mechanism for the changes in the electrocardiogram that occur with standing are great. They are mainly due to the fact that some investigators were exclusively interested in the alterations which appeared immediately after assuming the erect position while others studied only those changes which occured after minutes of standing. There is agreement that the *immediate* changes are due both to the change of the position of the heart and to an altered contact between the heart and the neighboring structures which show different conductivity.[1,2,3] The alterations which appear later, *viz.*, during continued standing are attributed by some authors to an altered blood supply to the heart,[4] while others assume a change in the tonus of the sympathetic nervous system which modifies the repolarization processes in the heart. This latter contention is based chiefly on the finding that after administration of sympathicolytic drugs positional changes failed to appear in the electrocardiogram.[5,6]

We studied this problem on 80 male patients who had no evidence of organic heart disease. Electrocardiograms were taken with the patient in the supine position, immediately upon standing, after standing for 1 minute, 5 minutes, 15 minutes and again immediately upon lying supine. In 12 patients 0.5 mg of Dihydro-ergotamine 45 was given intravenously and records were again taken in the supine position at the height of the systemic effects and on standing.

Results. Of the 80 patients, 25 or 31.2% showed significant electrocardiographic changes on standing. Some of the changes occurred immediately upon standing while others took place many minutes after standing was maintained.

Fig. 1 and Fig. 2 show that the final deflection in the electrocardiogram may become higher or lower with prolonged standing. Changes may appear immediately, disappear later and reappear (Fig. 1) or they may be absent immediately on standing, but appear later (Fig. 2).

A further significant result of our studies was the temporary appearance of A-V rhythm with a change of position in 4 cases (Fig. 3) and the frequent appearance of a marked sinus arrhythmia.

Finally, the studies revealed that in 11 out of 12 cases the significant electrocardio-

* The expenses of this study were defrayed by a grant from Bernhard Altmann.

1 Sigler, L. H., *Am. Heart J.*, 1938, **15**, 146.

2 Scherf, D., and Weissberg, J., *Am. J. M. Sc.*, 1941, **201**, 693.

3 White, P. D., Chamberlain, F. L., and Graybiel, A., *Brit. Heart J.*, 1941, **3**, 233.

4 Akesson, S., *Upsala läkeref. förh.*, 1936, **41**, 383.

5 Nordenfelt, O., *Acta med. scand. Suppl.*, 1941, **119**, 1.

6 Wendkos, M. H., *Am. Heart J.*, 1944, **28**, 549.

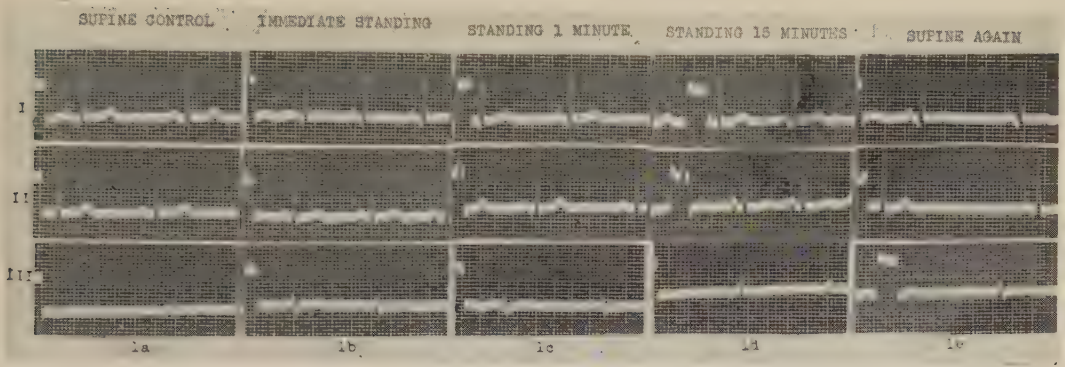

FIG. 1.

30-year-old healthy man; the changes which appeared immediately on standing disappeared within one minute; some alterations are again visible after standing for 15 minutes; on resumption of the supine position a marked bradycardia occurred and the electrocardiogram did not return to normal. Blood pressure varied between 105/70 and 120/70.

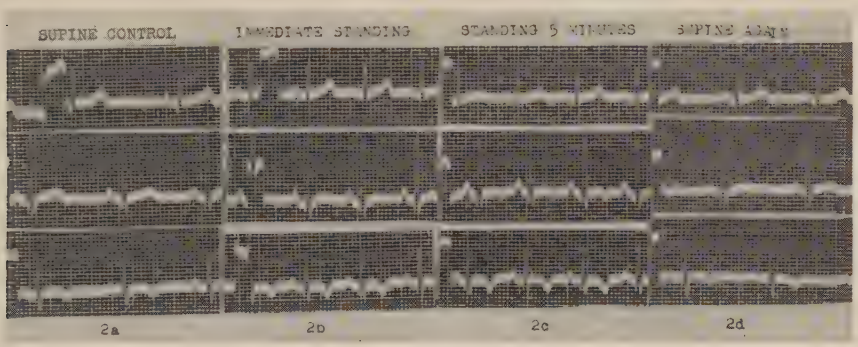

FIG. 2.

25-year-old man with early pulmonary tuberculosis; the alterations after standing for 5 minutes are more pronounced than those which appeared immediately on standing (lead II). On lying supine the blood pressure was 115/75, on standing it was 95/75.

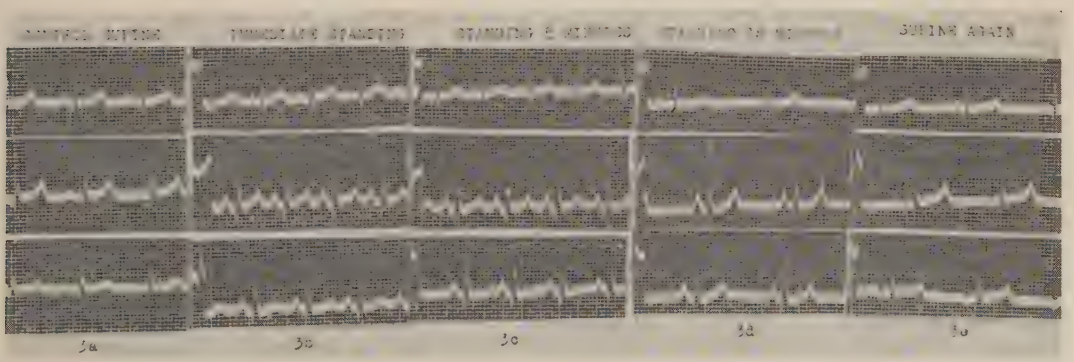

FIG. 3.

46-year-old man with early bronchogenic carcinoma. Changes appeared after 5 minutes of standing, and disappeared later (lead III); an A-V rhythm appeared after 15 minutes temporarily (lead I) and persisted for a few minutes on resumption of the supine position. No significant changes of blood pressure.

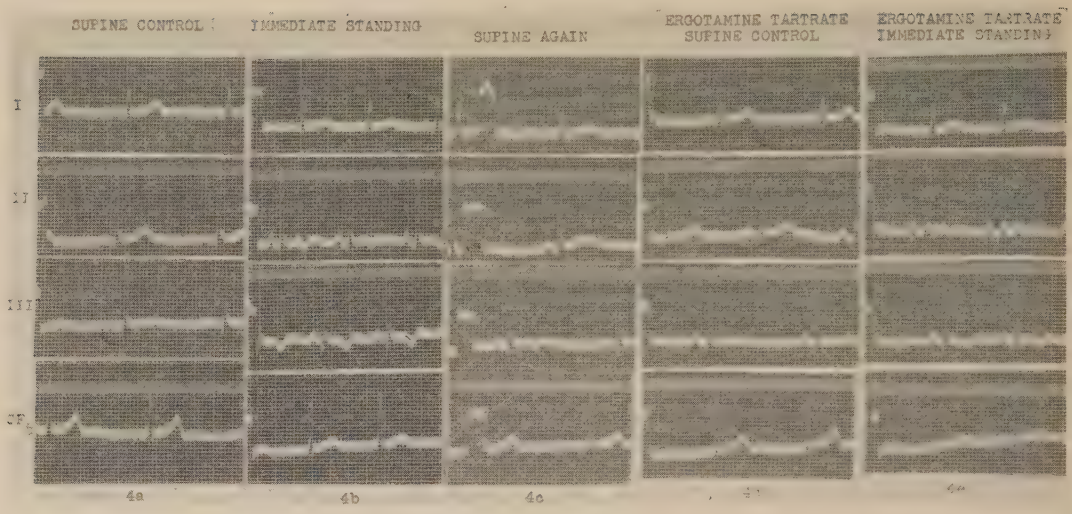

FIG. 4.
A 20-year-old man with intestinal parasites. Marked changes appear on standing and they remain uninfluenced by administration of ergotamine. No significant changes of blood pressure.

graphic changes which appeared immediately on standing could not be prevented by the administration of dihydro-ergotamine 45 (Fig. 4).

Comments. The *immediate* alterations of the electrocardiogram with changes of posture cannot be explained by an insufficient blood supply to the heart. In experiments in which the patients were tilted, it was shown that the changes appeared immediately when a certain angle was reached.[7]

The changes in the electrocardiogram caused by anoxia develop slowly within one or more minutes and also disappear gradually. The same argument holds for changes caused by a change in the tonus of the sympathetic nervous system.

Anoxia however, may be the explanation for those changes which occur during prolonged standing. Originally it was assumed[4] that prolonged standing leads to an accumulation of blood in the lower part of the body and a consequent diminished blood supply to the heart (orthostatic anemia). Unusually marked alterations in the limb and chest leads with simultaneous anginal pain were described in a healthy 21-year-old individual when in the erect position.[8] Against this interpretation for the electrocardiographic changes is the fact that in such patients the blood pressure may remain normal on standing, while in others with marked orthostatic hypotension no electrocardiographic changes appear.[5]

Another mechanism however, seems possible. Without the activity of certain reflexes marked hypotension and fainting would appear in every individual on standing. It is entirely possible that in the course of the widespread reflex regulation of the arterial bed the coronary vessels are involved. Marked changes in the electrocardiogram are observed in patients suffering from an acute profuse hemorrhage. These patients need not show any fall of blood pressure nor an anemia, since the alterations are observed with a hemoglobin of 80%. It has been concluded that reflexes from the carotid sinus and the McDowall reflex lead to a readjustment of the arterial bed in which the coronary arteries participate.[9] Thus reflex changes of the tonus of the autonomic nerves regulating the width of the coronary arteries in conjunction with the increased heart rate on standing may,

[7] Mayerson, H. S., and Davis, W. D., *Am. Heart J.*, 1942, **24**, 593.

[8] Akesson, S., *Acta med. Scand. Suppl.* 1947, **196**, 192.

[9] Scherf, D., and Klotz, S. D., *Ann. int. Med.*, 1944, **20**, 438.

temporarily lead to varying degrees of anoxia of the heart muscle.

The changes in the tonus of the autonomic nerves (in mammals particularly the sympathetic nerves) which has been mentioned above can also directly influence the final deflection of the electrocardiogram. In favor of the presence of such changes in tonus is the appearance of A-V rhythm with changes in posture (Fig. 3) and the remarkable fact that pronounced abnormalities of the P-R interval disappear or diminish when the subject is erect.[10,11,12] A change of the tonus of the sympathetic nervous system will modify the form of the T waves and the ventricular gradient. The sympathetic nerves accelerate the repolarization process. Under normal conditions the subendocardial layers are depolarized earlier and repolarized later than the subepicardial muscular layers. This is probably due to the high intraventricular pressure. A faster repolarization of the inner layers would lead to a depression and inversion of the T waves.

The importance of a change in the tonus of the sympathetic nervous system is demonstrated by the absence of positional electrocardiographic alterations after the administration of sympathicolytic drugs.[5,6,13] The frequent ineffectiveness of ergotamine shown in this report (Fig. 4) had to be expected, because the immediate changes appearing on standing are not due to a change of sympathetic tonus as frequently maintained. There is furthermore no proof that the sympathetic system alone is involved in the electrocardiographic changes which appear on continued standing.

Summary. The immediate alterations in the eletrocardiogram caused by assuming the erect posture and those appearing during standing, were studied in 80 patients.

The genesis of the changes which appear immediately on standing and disappear immediately when the supine posture is resumed is separated from those which are seen during prolonged standing. The latter is ascribed to changes in the tonus of the autonomic nervous system which may act on the heart muscle directly or via the coronary arteries.

A-V rhythm may appear on standing due to the change of the tonus of the autonomic nerves.

Administration of ergotamine preparations does not always prevent the appearance of immediate changes in the electrocardiogram on change of posture.

Our thanks are due to Dr. Henze of the Sandoz Drug Company for the supply of Dihydro-Ergotamine 45.

[10] Poel, W., *Arch. int. Med.*, 1942, **69**, 1040.

[11] Holmes, J. H., and Weill, D. R., Jr., *Am. Heart J.*, 1945, **30**, 291.

[12] Manning, G. W., and Stewart, C. B., *Am. Heart J.*, 1945, **30**, 109.

[13] Spuehler, O., *Schweiz. med. Wchschr.*, 1947, **77**, 28.

16420

A Comparison of the Acute Toxicity of Two Forms of Thiamine.*

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Recently there has become available for investigational use a new form of vitamin B_1, thiamine mononitrate. Considering the wide therapeutic use and the parenteral toxicity of thiamine hydrochloride,[1-13] it was thought

* Present address: Department of Pharmacology and Toxicology, University of Southern California School of Medicine, Los Angeles, Calif.

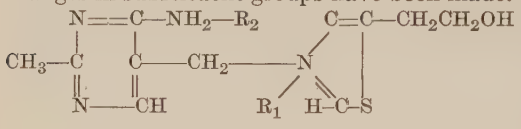
[1] Steinberg, C. L., *Am. J. Digestive Dis.*, 1938, **5**, 680.

TABLE I.
Properties of Two Forms of Thiamine.

Property	Thiamine Hydrochloride	Thiamin Mononitrate
Melting point	245-248°C	196-200°C dec.
Molecular wt	337.26	327.36
Units per mg	333	343
Solubility	100 g per 100 g water	2.7 g per 100 g of water

advisable to compare the hydrochloride and the mononitrate in regard to their toxicity in animals.

Comparison of the structural formulas of both forms of thiamine shows where the changes in substituent groups have been made.



	R_1	R_2
Hydrochloride	Cl	HCl
Mononitrate	NO_3	—

Table I shows the chemical and physical characteristics of both compounds.[14-15] From these data one can conclude that, although the 2 forms are approximately equal in potency, their solubility characteristics would favor the use of the hydrochloride when large doses are to be administered parenterally. However, the solubility of the mononitrate increases to 18.5 g per 100 cc when the pH of the solutions is adjusted to 4.0. Aqueous solutions at this pH are stable for one year,[14] while aqueous solutions of the hydrochloride at pH 2.7 to 3.0 begin to show a loss of potency in about 6 months.[16]

Acute toxicity studies of thiamine hydrochloride have shown that, although large oral doses were toxic, these doses were much larger than those used therapeutically.[17-20] However, the same is not true of parenterally administered doses of the drug. Early studies indicated toxic symptoms both in animals and humans[1,2,18-20] and recently Haley and Flesher[21] found that rabbits developed symptoms of toxicity after intravenous injections of 200 to 300 mg per animal. This work has been extended to dogs by Smith *et al.*[22] who have reported similar results.

Experimental. Acute toxicity of the hydrochloride was determined by intraperitoneal injection in mice and intravenous injection in rabbits and of the mononitrate by intraperitoneal and intravenous injection in mice and intravenous injection in rabbits. In the mouse experiments, a total of 150 animals weighing 22-42 g were used and the concentration of the drugs was 50 mg/cc. The dosage ranged from 0.04 to 0.07 cc intravenously and from 0.17 to 0.32 cc intraperitoneally. Death occurred within 5 minutes after intraperitoneal and within 30 seconds after intravenous injection in mice. In the rabbit experiments

2 Stern, E. L., *Am. J. Surg.*, 1938, **39**, 495.

3 Steinberg, C. L., *J. Am. Med. Assn.*, 1941, **116**, 2713.

4 Laws, C. L., *ibid.*, 1941, **117**, 176.

5 Schiff, L., *ibid.*, 1941, **117**, 609.

6 Stiles, M. H., *ibid.*, 1941, **117**, 954.

7 Stiles, M. H., *J. Allergy*, 1941, **12**, 507.

8 Mills, C. A., *J. Am. Med. Assn.*, 1941, **116**, 2101.

9 Kalz, F., *J. Invest. Dermat.*, 1942, **5**, 135.

10 Eisenstadt, W. S., *Minnesota Med.*, 1942, **25**, 861.

11 Leitner, A. A., *Lancet*, 1943, **2**, 474.

12 Stein, W., and Morgenstein, M., *Ann. Int. Med.*, 1944, **20**, 826.

13 Reingold, I. M., and Webb, F. R., *J. Am. Med. Assn.*, 1946, **130**, 491.

14 Merck and Co., *Thiamine Mononitrate*, January 1947.

15 Merck and Co., *Service Bulletin and Vitamin B_1*, May 1943, pp. 1-2.

16 Haley, T. J. Unpublished results.

17 Molitor, H., *Fed. Proc.*, 1942, **1**, 309.

18 Hecht, G., and Weese, H., *Klin. Wchschr.*, 1937, **16**, 414.

19 Perla, D., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 169.

20 Molitor, H., and Sampson, W. L., *Merck Jahresbericht*, 1936, **50**, 51.

21 Haley, T. J., and Flesher, A. M., *Science*, 1946, **104**, 567.

22 Smith, J. A., Foa, P. P., and Weinstein, H. R., *Fed. Proc.*, 1947, **6**, 204.

TABLE II.
Toxic Doses of Two Forms of Thiamine.

Animal	Form	Route of administration	Dose mg/kg	Mortality ratio	LD_{50} mg/kg	Standard error
Mouse	Mononitrate	Intravenous	80	0/5	84.24	±1.14
			84	4/5		
			86	3/5		
			88	3/5		
			90	3/5		
			92	5/5		
"	"	Intraperitoneal	380	1/5	387.3	±1.65
			385	3/5		
			390	2/5		
			395	4/5		
			400	3/5		
"	Hydrochloride	"	310	3/5	329.8	±3.93
			320	3/5		
			330	3/5		
			335	4/5		
			340	4/5		
			350	4/5		
			Animal wt, kg	Total dose, mg	Intrav. lethal dose, mg/kg	Avg intrav. lethal dose, mg/kg
Rabbit	Mononitrate	Intravenous	3.66	500	136.61	112.58
			4.30	475	110.46	
			3.22	375	116.46	
			4.44	437.5	98.53	
			4.66	470	100.85	
"	Hydrochloride	"	1.704	180	105.63	117.45
			1.818	220	121.01	
			1.591	200	125.70	

the dosage was 1 cc (50 mg/cc) per minute until death, which occurred within 10 minutes. The rabbits used weighed 3.22 to 4.44 kg. Table II gives the mortality figures for mice including the LD_{50} which is the dose calculated to kill 50 per cent of the mice, according to the method of Miller and Tainter;[23] Table II also lists the intravenous dose required to kill all of the rabbits injected.

The symptoms of toxicity observed were: restlessness, labored respiration, vasodilatation, cyanosis, muscular twitching, clonic convulsions and death by respiratory paralysis. This respiratory paralysis was of central origin because electrical stimulation of both the muscle of the diaphragm and the phrenic nerve showed that the muscle was still capable of contraction. Visual signs of anoxia were a gradually deepening bluish coloration of the ears and all other body areas where the fur was thin enough to permit direct observation of the skin. In all animals cardiac arrhythmias were seen upon opening the thoracic cage. Auricular/ventricular rates of from 2:1 to 5:1 were common.

In conjunction with these toxicity studies more than 100 unanesthetized rabbits, weighing between 3.11 and 5.33 kg, were injected intravenously with solutions of thiamine hydrochloride ranging from 10 to 100 mg/cc. This routine testing over the period of one year showed that, although fatalities were seldom observed, clonic convulsions were produced in 80% of the animals when the total dose was above 300 mg per animal. These convulsions usually occurred after the animal had been returned to its cage. No visual signs of anoxia, cyanosis of the blood in the ear veins or of the skin in the scrotal region, were seen. However, no blood samples were

[23] Miller, L. C., and Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 261.

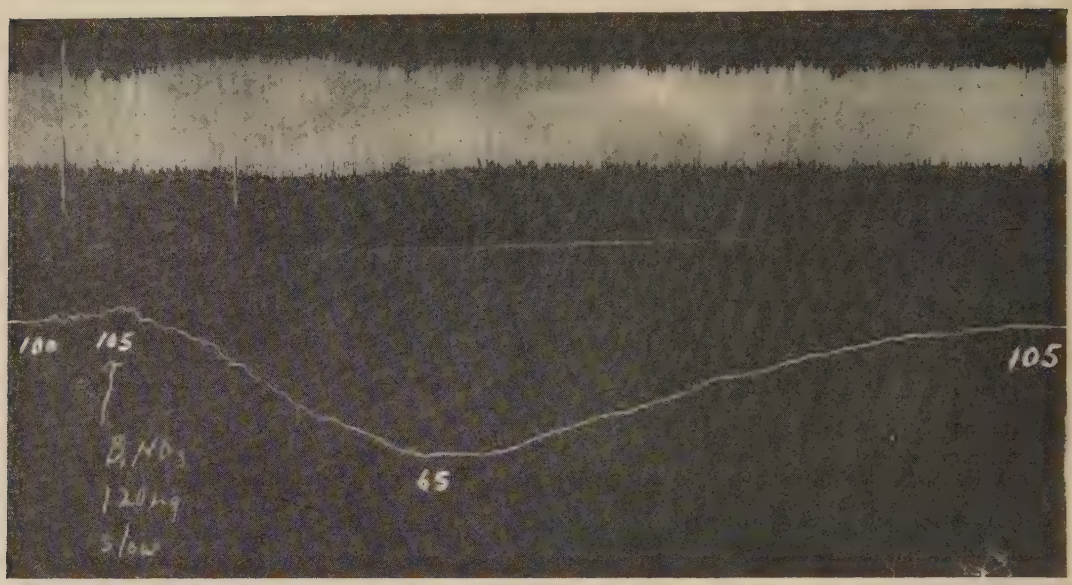


FIG. 1.
Top, Respiration. Bottom, Blood Pressure.

obtained for the determination of oxygen content so that partial anoxia cannot be entirely ruled out as a causative factor in the convulsions.

Inasmuch as Molitor[20] has shown that thiamine has a pronounced effect upon respiration and little effect upon the blood pressure of the dog, it was decided that a study of this phenomenon should be undertaken. Six rabbits, weighing 3.66 to 5.22 kg were anesthetized with 20 mg/kg of sodium pentobarbital intravenously and 3.5 cc/kg of 20% urethane intraperitoneally and prepared for recording of blood pressure via the carotid artery and respiration by cannulation of the trachea. Respiration was recorded with the Haley respirometer[24] and blood pressure with a mercury manometer. The two forms of thiamine in a concentration of 50 mg/cc in normal saline were injected at a fixed dose of 120 mg alternately. As the rate of injection partially determines the effect on the animal, 2 injection rates were employed; slow (1 mg/second) and fast (12 mg/second). Further, in order to rule out the effect of pH, 2 rabbits were injected with the mononitrate at pH 6.8 and 4 at pH 0.9. The hydrochloride was always at pH 2.7. Fig. 1 shows a typical record of a slow injection and Fig. 2 is typical record of a fast injection.

The results of this work show that slow injections of either form of thiamine had very little effect on respiration but caused a fall in blood pressure averaging 36 mm of mercury. This fall was gradual, requiring 114 seconds to be completed and the recovery required 219 seconds to reach the previous normal level. Fast intravenous injections had a more pronounced effect on respiration, decreasing both the rate and the depth. There was also a blood pressure fall averaging 23 mm of mercury.

Discussion. From the results of the acute toxicity determinations given in Table II there appears to be little difference in the toxic doses of either form of thiamine. Further, Molitor[25] has found that the mouse intravenous toxicity of the hydrochloride is 85 mg/kg which agrees with the 84.24 mg/kg figure for the mononitrate.

From the results herein presented as well as those of previous investigators[20–22] one must conclude that thiamine exerts its principal toxic effect by paralysis of the respiratory center. However, the work of Zaidi[26] on the

[24] Haley, T. J., *J. Am. Pharm. Assn.*, in press.

[25] Molitor, H., personal communication.

[26] Zaidi, S. H., *Ind. Med. Gaz.*, 1947, **82**, 181.

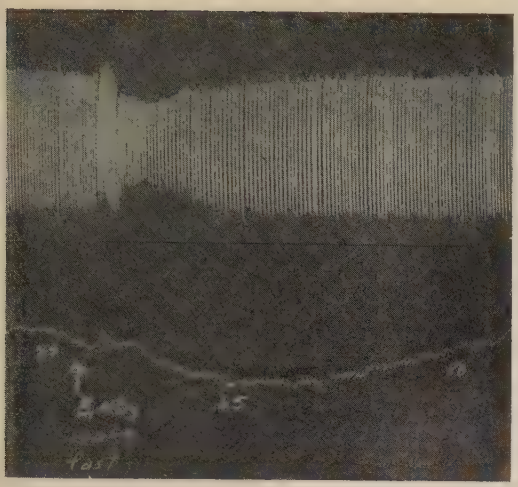


FIG. 2.
Top, Respiration. Bottom, Blood Pressure.

isolated frog heart and Smith *et al.*[22] on the isolated turtle heart indicates that thiamine has also a direct toxic effect on the myocardium. Furthermore, irregularities in the electrocardiogram of dogs after the administration of large doses of thiamine[22] shows that the mammalian heart is affected.

Smith *et al.*[22] found that, even after vagotomy or atropinization, there was a prolonged peripheral blood pressure fall. Haimovici and Pick[27] reported that thiamine caused vasodilatation which counteracted the vasoconstrictor action of nicotine on the perfused frog hind limb preparation. Thus it is probable that thiamine causes vasodilatation by direct action on the peripheral vascular musculature.

Summary. There is little difference in the lethal dose of either thiamine hydrochloride or mononitrate and the symptoms of toxicity are the same for both forms of the drug. The toxic effects of both forms of the vitamin on respiration and blood pressure are due to their thiamine content and not to the pH of the injected solutions or to the substituent groups on the nitrogen of the thiazole nucleus. In lethal doses either form of the vitamin causes death by a direct paralyzing action on the respiratory center followed by cardiac failure.

The author wishes to thank Merck and Co. for the generous supply of thiamine mononitrate used in this study.

27 Haimovici, H., and Pick, E. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 234.

16421

Influence of Nephrectomy and Renal Pedicle Ligation on the Activity of Liver Arginase in Rats.*

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Intermediary nitrogen metabolism has been reported to be impaired in dogs with reduced or abolished kidney function.[1] This deficiency is manifest in the fact that 30 to 75% of fed nitrogen is not transformed into urea.

Consideration was given to the possibility that renal transformation of citrulline into arginine[2,3] might represent a reserve mechanism for the urea cycle in the liver and that curtailment of this renal reserve might be related to the observed deficiency.

The hypothesis that an increase in arginase activity might compensate—at least in part—for the lesser concentration of arginine and ornithin by speeding up the turnover of the urea cycle led to a series of experiments. They include determinations of liver arginase

* Aided by a grant from the Commonwealth Fund.

† James Hudson Brown Junior Fellow.

1 Mylon, E., Smith, E. R., and Goldstein, P., *Am. J. Physiol.*, 1948, in press.

2 Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, 1941, **141**, 717.

3 Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, 1946, **166**, 239, 251.

activity in normal, fasting, nephrectomized and renal artery ligated rats.

The results are contained in the report that follows:

Materials and Methods. Male albino and black hooded rats weighing from 250 to 400 g were standardized on a Purina Chow diet.

Removal of the kidneys as well as ligation of the renal pedicles was performed through a midline abdominal incision under light nembutal anesthesia (4 mg/100 g wt). Appropriate sham operations were carried out in all normal rats prior to the fasting. Nephrectomized animals and renal artery ligated animals were fasted after the operative procedures.

Liver arginase activity was determined by a slight modification of the method of Hunter and Downs.[4] The procedure follows:

The livers of lightly nembutalized rats were removed and weighed. In many instances a small piece of liver was dried to constant weight at 110° C. The remainder was placed in a Waring blendor with 50 ml of ice cold normal saline.

Homogenization was carried out in 2½ minute periods. After the first of these, the glass container was cooled in a deep freezer for 7 to 8 minutes so that the temperature of the homogenate did not rise above 40° C during the entire 5 minute procedure. A high speed homogenizer designed for 50-70 ml was used.

Aliquots of 5 ml of the homogenate were activated, diluted and incubated with a buffered arginine solution.

Urea determinations were made by the urease-aeration method of Van Slyke and Cullen;[5] commercial urease "Arlington" was used. All calculations were based on the "arginase unit" as described by Hunter and Downs.[4]

Results. Normal rats. The highest liver arginase activity in 18 normal rats was 300 units per g wet weight, the lowest, 233 units and the average 268 ± 23.1‡ units. The average arginase activity in the total liver was 3605 units. The relationship of liver weight to body weight and liver dry weight to wet weight was rather uniform; 3.73% of the body weight was the average weight of the liver and 28.6% of the wet weight was the average dry weight.

Fasting normal rats: After 48 to 72 hours of fasting the liver arginase activity had only slightly decreased; the highest value in 12 animals was 290.5 units/g wet weight, the lowest 222.1 and the average 256 ± 25.9‡ units. The marked decrease in liver weight on fasting, 32.4%, was the main factor, therefore, in the reduction of the total liver arginase to 2375 units.

Nephrectomized rats: Arginase activity in liver, 48 hours after bilateral nephrectomy was in the range of that of normal fasting rats averaging 261.5 units per g wet weight.

In contrast, 72 hours after nephrectomy a marked increase of the arginase activity was found; the highest value in 8 experiments was 447 units per g wet weight, the lowest 331 units, and the average 373.7 ± 46.2‡ units. This increase observed in nephrectomized fasting rats as compared to the controls, *i.e.* normal fasting rats, is highly significant (p value <0.001, Fisher's tables).

While the percentage of dry weight to wet weight in the liver was the same as in normal rats, its decrease in weight was only 22% during the 72 hours following the nephrectomy as compared with a loss of 32.4% observed in the normal group fasted for the same period. The high value for total liver arginase, 3487 units, obviously is based on two additive factors: One the significant increase of arginase units per g wet weight, and the other, the smaller loss in the liver weight after nephrectomy.

Rats with bilaterally ligated renal pedicles: The severity of this intervention, indicated by the death of all animals of the first series before the end of the second day required termination of the experiments 24 hours after ligation. Despite this short experimental period the arginase units per g wet weight

[4] Hunter, A., and Downs, C. E., *J. Biol. Chem.*, 1944, **155**, 173.

[5] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, **19**, 211; *J. Biol. Chem.*, 1916, **24**, 117.

‡ Standard deviation of the mean.

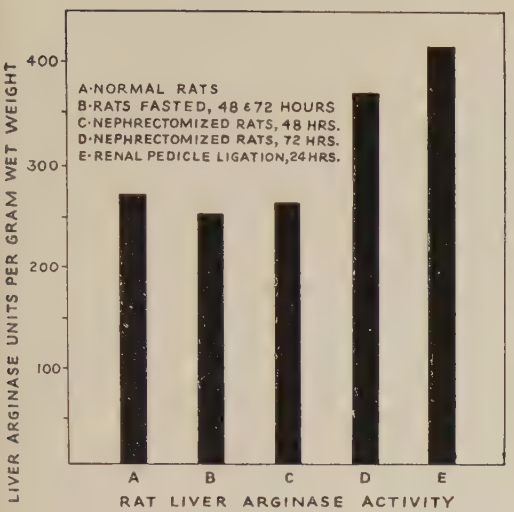

FIG. 1.

Comparison of liver arginase activity of normal, fasting, and nephrectomized rats, and rats after bilateral renal pedicle ligation.

were extremely high, 466.4 units as a maximum, 351.5 as a minimum, with an average of 412.4 ± 51.6 units.§ Fig. 1 is a survey of the results obtained.

Discussion. Liver arginase activity of normal rats on a high protein diet,[6] of hypophysectomized and of adrenalectomized rats injected with cortical steroids[7-9] has been reported to be markedly elevated. The question arises whether the change in liver arginase activity as reported in this paper is related to a similar increase in nitrogen metabolism. This has been found not to be the case. The increase in blood urea-N on the third day following nephrectomy would indicate that only 1.438 g of protein were metabolized¶ by a 250 g rat, an amount which corresponds to less than one half of the daily intake on Purina food. The nitrogen metabolism of a normal rat on this diet, therefore, is significantly higher than that of a fasting nephrectomized rat. This makes it difficult to assume that the increased liver arginase activity following removal of the kidneys is associated with an elevation of the nitrogen metabolism.

The possibility is considered that elimination of kidney function limits one or several components of the urea cycle, and that under these circumstances, increase in arginase activity may be a compensatory mechanism. It must be emphasized, however, that the deficiency in liver arginine or ornithin in the above conditions is an assumption.

Summary. Liver arginase activity is significantly increased after bilateral nephrectomy and also after ligation of the renal arteries in rats. This reaches a maximum within 24 hours after ligation of the arteries as compared with 72 hours after removal of the kidneys. The possibility is considered that the marked increase in arginase activity is a compensatory mechanism for a deficiency in renal arginine formation.

§ Standard deviation of the mean.

[6] Lightbody, H. D., and Kleinman, A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 472.

[7] Fraenkel-Conrat, H., and Evans, H. M., *Science*, 1942, **95**, 305.

[8] Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, 1943, **147**, 99.

[9] Folley, S. J., and Greenbaum, A. L., *Biochem. J.*, 1946, **40**, 46,

¶ Total urea formation was calculated from the increase of blood urea per day, assuming it is equally distributed in body water (75% of the body weight).

16422

Inability of 2-3 Dithiopropanol* (BAL) to Protect the Liver Against Toxic Action of Uranium Nitrate.

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In a recent issue of this journal[1] certain observations were presented concerning the hepatoxic action in normal dogs of 2-3 dithiopropanol. This investigation was closed by the following conclusion: "The experiments indicate the ability of a dithiol when given to normal dogs in an appropriately large amount per kilo to induce an hepatic injury which is characterized by fatty degeneration of the hepatic epithelium and rarely by a necrosis of this tissue. Such observations do not necessarily imply that a similar order of cellular injury would be induced in the dog under the influence of an intoxication by salts of certain of the heavy metals. Such bodies offer a bond of union for the dithiols which in turn may prevent a toxic effect which they are capable of inducing in tissues of normal constitution."

In order to examine into the above assumption the following experimental procedures have been undertaken. Eight adult dogs have been given subcutaneously one injection of 4 mg of uranium nitrate per kg. As has been previously demonstrated[2] the hepatic injury from such a dosage of uranium nitrate usually reaches its height between the sixth and eighth day of the intoxication. All of the animals survived the period of experimentation. With this group of control animals biopsy material was obtained from the liver on the seventh day of the intoxication for purposes of histological study. Such tissue shows a diffuse type of degenerative process in the lobules without any tendency for the injury to be initially localized in the lobular structure. The hepatic epithelium is increased in volume from an accumulation of granules of an assumed protein derivation and by granules and droplets of lipoid material which give the characteristic microchemical reaction with Scharlach R. Such droplets fuse to form larger areas of lipoid material with a displacement of the degenerating cell nuclei. Areas of extensive necrosis have not been observed.

A second group of 8 normal dogs have been intoxicated by the subcutaneous administration of 4 mg of uranium nitrate per kg. At the time of such an injection these animals were also given intramuscularly 15 mg of 2-3 dithiopropanol per kg. The dithiol injections were continued at 9 A.M. and 9 P.M. during the course of the uranium nitrate intoxication. Five of the animals of this group failed to survive the period of 8 days allowed for the experiments. The remaining 3 dogs were sacrificed on the eighth day. Liver tissue from these animals has shown an intensification of those changes which are induced in the liver when uranium nitrate constitutes the only toxic factor employed during the experiments. (Fig. 1.) The hepatic epithelium shows a diffuse necrosis. The liver cells are outlined by accumulations of lipoid material which in some instances has ruptured the cell membrane to fuse with such material in adjacent cells. The cell nuclei have disappeared as a part of the process of necrosis.

Conclusion. In the doses employed (15 mg per kg, twice daily for 8 days), 2-3 dithiopropanol was ineffective in protecting the liver of dogs against uranium nitrate in the amounts administered.

* 2-3 dithiopropanol (BAL), 10%, benzyl benzoate 20% in peanut oil.

1 MacNider, W. deB., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 444.

2 MacNider, W. deB., *J. Pharm. and Exp. Therap.*, 1936, **56**, 359.

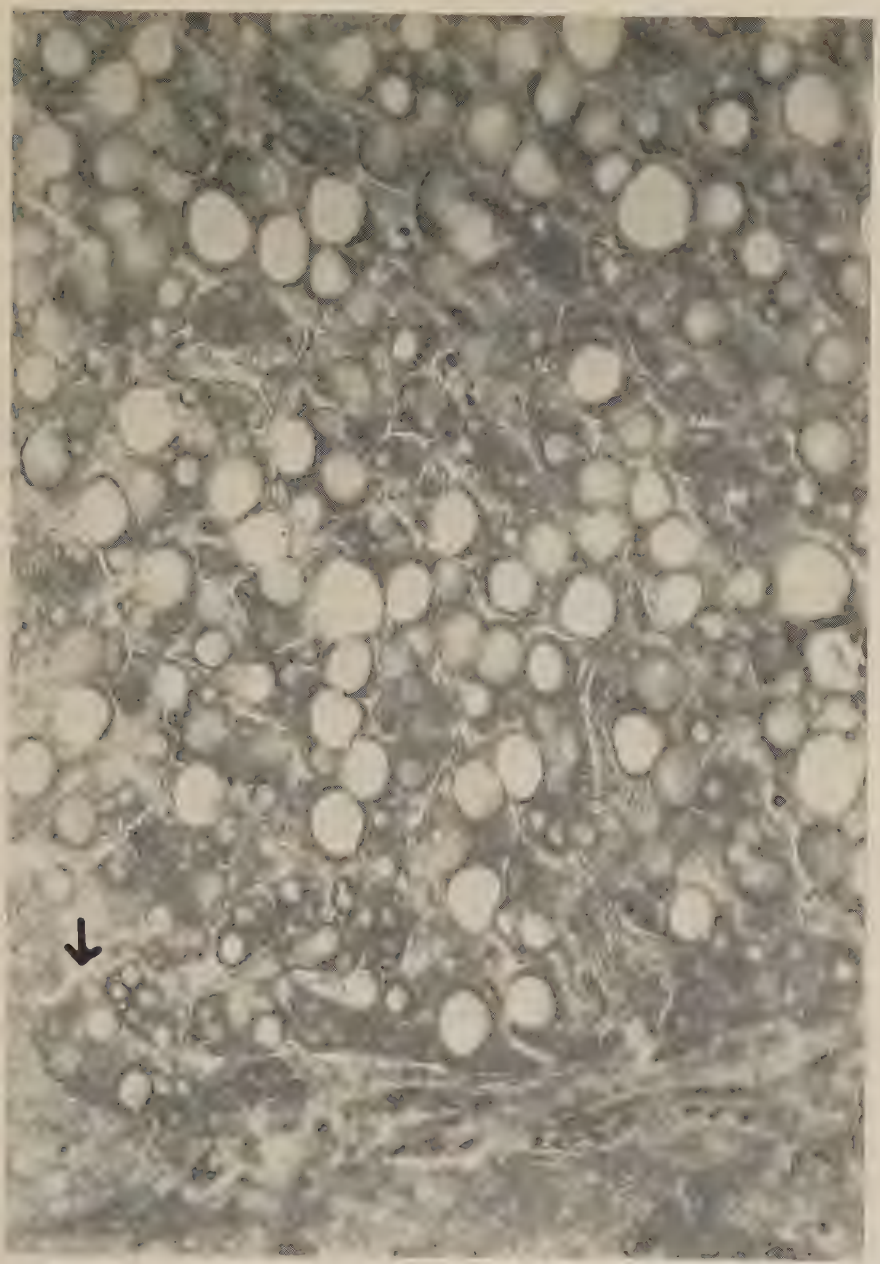

Fig. 1.

Microphotograph, Zeiss × 395, hematoxylin and eosin. The figure is from the liver of animal 6, sacrificed on the eighth day of a uranium nitrate intoxication during which period the animal had received daily two intramuscular injections of 15 mg of 2-3 dithiopropanol. The figure indicates a diffuse order of hepatic necrosis with advanced fatty degeneration. The lower portion of the figure shows as outlined by fragments of nuclear material a collapsed interlobular vessel.

16423

A Precipitin Test for Antigens Present in Mouse Tissues Containing the Milk Agent.*

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(Introduced by John J. Bittner).

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One of the causes of mouse mammary carcinoma was shown by Bittner[1] to be an agent transmissible through the milk of nursing females to their young. Investigations on the nature of this agent show that its size is within the realm of viruses, as demonstrated by Visscher, Green, Bittner, Ball, and Siedentopf.[2] The antigenic character of the milk agent was described by Andervont and Bryan,[3] who demonstrated the presence of specific neutralizing antibodies in the sera of rabbits inoculated with extracts of tumor tissues, while Green, Moosey, and Bittner[4] showed that highly active specific antisera, which would neutralize the agent, could be produced in rabbits and rats.

The first successful attempt to identify the milk agent by an *in vitro* serological method was described by Gerodilova and Shabad,[5] who used a complement fixation test, and more recently by Bennison,[6] who used similar methods.

In our studies on the antigenic nature of the milk agent, we have been able to demonstrate that when rabbits are injected with centrifugates of mouse tissues containing the agent, sera are produced which specifically precipitate ultramicroscopic elements derived from cancer tissues and other agent-bearing tissues of mice. The test to be subsequently described will therefore be referred to as a precipitin test and the serum antibodies as precipitins.

Materials and Methods. Adenocarcinomas of the mouse mammary glands, which contain relatively large amounts of milk agent, were selected as a source of antigen. The cancerous tissues were first ground in a mortar and diluted approximately 1-10 by weight in physiological saline. The suspensions were then passed through a homogenizer to further break up the tissue cells. This homogenate was centrifuged at 2,000 R.P.M. for 30 minutes at 4° C. The sediment, consisting of unbroken cells and large particles, was discarded, while the supernatant fluid was again centrifuged at 15,000 R.P.M. in an angle centrifuge for one hour at 4° C. The resulting pellet of centrifugate was suspended in saline so that 1 cc contained the centrifugate derived from one g of tumor tissue. Rabbits received this suspension as antigen in five intramuscular injections of 1 cc at 5 day intervals. Two weeks after the final injection sera were collected and utilized as immune sera.

The antigens used in the precipitin test were prepared similarly to those used for rabbit inoculation, except that the final suspension contained the centrifugate derived from 1 g of tissue in 3 cc of diluent. The centrifugate was carefully put into suspension by mechanical agitation and cleared by recentrifuging at 1,000 R.P.M. for 30 minutes.

* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research and The Graduate School of the University of Minnesota.

1 Bittner, J. J., *Science*, 1936, **84**, 162.

2 Visscher, M. B., Green, R. G., Bittner, J. J., Ball, Z. B., and Siedentopf, H. A., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 94.

3 Andervont, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1944, **5**, 143.

4 Green, R. G., Moosey, M. M., and Bittner, J. J., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 115.

5 Gerodilova, V. V., and Shabad, L. M., *Bull. Eksp. Biol. i. Med.*, 1946, **22**, 9.

6 Bennison, B. E., Abstract, Fourth International Cancer Research Congress, September 2-7, 1947, St. Louis, Mo.

The actual quantity of antigen used in the precipitin tests was determined accurately by the micro-Kjeldahl technic. Analyses were conducted by using a steam distillation apparatus and the ammonia was collected in 2% boric acid solution containing methyl red.

Antigens were prepared from mouse cancer tissue, non-lactating mouse mammary gland containing the agent, non-lactating mammary gland without the agent, pooled spleen, heart, liver and lung containing the agent, and similar tissues without the agent. Sera were collected from rabbits immunized against mouse cancer antigen and antigens derived from non-lactating mammary glands with and without the agent.

The precipitin tests were carried out in the following manner. Antigen and antiserum were separately diluted in serial 2-fold dilutions. One-half cc of antigen dilution was added to ½ cc of antiserum dilution in Wassermann-type tubes. The antigen-antibody mixtures were then placed in an incubator at 37.5° C for half an hour. The tubes were then stored at 4° C for 18 hours. At the end of this time the settled granular precipitates were observed and read as 1+, 2+, 3+, or 4+, depending on the degree of precipitation. 4+ was the maximum precipitate observed, while the minimum definite precipitate was recorded as 1+.

Precipitin Tests. Antiserum prepared with mouse cancer antigen was tested against antigens derived from mouse cancer tissue, non-lactating mammary glands with and without the agent, and pooled spleen, heart, liver, and lung with and without the agent. In no case did precipitation occur when normal rabbit serum was mixed with various antigens. The results, presented in Table I show that antiserum against mammary cancer antigens will precipitate in high serum dilutions normal mammary gland tissue and pooled organ tissues containing the agent, while the same antiserum will precipitate normal mammary gland tissue and pooled organ tissues without the agent only in low serum dilutions. These precipitations occurring in low serum dilutions are best explained on a basis of normal tissue occurring within the masses of cancer tissue.

TABLE I.

Precipitin Reactions of Specific Sera Against Milk Agent Derived from Different Tissues.

Antigen	Antiserum	Serum dilutions									
		U	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
*Mouse cancer	Mouse cancer	++++	+	+	+++	+ ±	+	+	+	+	±	
* " "	Gland without agent	+	++	++	+	+	+	+				
* " "	Gland with agent	++	++	+	+							
*Gland with agent	Mouse cancer	++	++	++	+	+	++	++	++	±		
*Gland without agent	" "	+	+	+	+	+	+					
†Tissue with agent	" "	++	++	++	+	++	±	+				
†Tissue without agent	" "	+	++	++	±							

* 1:45 dilution.
† 1:60 dilution.

It is not to be expected that serological reactions involving antigenic preparations made from cancerous tissues, such as those reported here, would be entirely devoid of cross reactions with normal mouse tissue. A mass of cancer tissue would appear to contain many normal tissue elements, such as blood vessels stimulated to grow into the cancer cell mass and normal tissue enclosed by the cancerous growth and not yet degenerated. A serum made by injecting antigens derived from such a mixture of elements would be expected to contain antibodies against both the cancer cell and the normal tissue derivatives.

Antisera prepared with non-lactating glands containing the agent and without the agent were then tested against antigens derived from mouse cancer tissue. As shown in Table I, antiserum against normal mammary gland tissue abundant with the milk agent will precipitate mammary cancer antigen in high serum dilutions. Opposite results are observed when the milk agent is absent in the normal mammary gland tissue. These results indicate that the specific antigen abundant in mouse mammary cancer tissues can also be observed in non-lactating mammary gland and organ tissues containing the agent and is absent in mammary glands and organ tissues lacking the agent.

The concentration of all the antigens used in this work is expressed in terms of the weight of the original tissue. For example, a dilution of 1-60 means that the centrifuged pellet obtained from 1 g of tissue is diluted in 60 cc of saline. It is evident from this that the concentration of the specific antigen will not be the same in similar dilutions from different tissue preparations unless the concentration of the antigen is the same in all these tissues. This is not likely to be the case.

If the antigens were pure, the concentration could be controlled by running nitrogen determinations and diluting the various preparations to the same organic nitrogen concentration. The control of the organic nitrogen concentration will not suffice in this case, because the various preparations may contain different percentages of organic nitrogen from sources other than the specific antigen.

It is a well-known fact that the amount or degree of precipitation following an antibody-antigen reaction is dependent upon the relative concentrations of antibody and antigen.

TABLE II.

Precipitin Reactions of Mouse Mammary Cancer Antiserum Against Tissues with and without the Milk Agent.

A.

Serum dilutions	Antigen dilutions				
	1/60	1/120	1/240	1/480	1/960
1/3	++	+	+	+	+
1/6	+	+	+	+	+
1/12	+	+	+	+	—
1/24	+	+	+	—	—
1/48	+	+	+	—	—
1/96	+	+	+	—	—
1/192	+	+	+	+	—
1/384	+	+	+	—	—
1/768	+	+	+	—	—

Antigen: Mouse Mammary Cancer, 1/60 dilution = .018 mg N/cc.
Serum: Mouse Mammary Cancer Antiserum.

B.

Serum dilutions	Antigen dilutions				
	1/60	1/120	1/240	1/480	1/960
1/3	+	+	+	+	+
1/6	+	+	+	+	—
1/12	+	+	+	+	+
1/24	+	+	+	—	—
1/48	+	+	+	—	—
1/96	+	+	—	—	—
1/192	+	—	+	—	—
1/384	—	+	+	—	—
1/768	+	+	—	—	—

Antigen: Mammary Gland Tissue with agent. 1/60 dilution = .009 mg N/cc.
Serum: Same as A.

C.

Serum dilutions	Antigen dilutions				
	1/60	1/120	1/240	1/480	1/960
1/3	+	+	+	—	—
1/6	+	+	+	—	—
1/12	+	+	—	—	—
1/24	+	+	—	—	—
1/48	—	—	—	—	—
1/96	—	—	—	—	—
1/192	—	—	—	—	—
1/384	—	—	—	—	—
1/768	—	—	—	—	—

Antigen: Mammary Gland Tissue without agent. 1/60 dilution = .007 mg N/cc.
Serum: Same as A.

In fact, if the ratio of antigen to antibody, or the reciprocal ratio of antibody to antigen is too great, the result may be no precipitation. It is for this reason that as long as the absolute concentration of antigen is unknown, 2 antigen preparations cannot be compared by trying each against several dilutions of the same serum. Thus, the results given on Table I may not be conclusive.

To overcome this objection, varying dilutions of the antigen were tried against varying dilutions of the antibody. The results of a titration of this kind are presented in Table II-A, II-B and II-C. Antigen controls in dilutions of 1-60 and greater were negative. Serum controls in all dilutions were also negative. As in previous experiments, normal rabbit serum did not react with any of the antigen. It is evident from a study of Table II that the zones of precipitation are similar and greater when the mouse mammary cancer antiserum is tested against mouse mammary cancer or mammary gland tissues with the agent (Table II-A and II-B) than when the same serum is tested against mammary gland tissues without the agent (Table II-C). This would indicate that there is a common antigen present in mouse mammary cancer and mammary gland tissues containing the agent and which is not present in the antigen prepared with the mammary gland tissues without the agent.

Adsorption Tests. The results so far indicate that in sera of rabbits injected with mouse mammary cancer centrifugates, antibodies against both the cancer cell and normal tissue derivatives are present. In order to broaden the observations, tests were carried out to eliminate the normal tissue antibodies by precipitin-adsorption reactions. When antigen derived from normal mammary gland tissues without the agent is added to the mouse cancer antiserum, a moderate precipitate will result after incubation (0.279 mg N of antigen to 1 cc of serum). Centrifugation at 3,000 R.P.M. for 30 minutes at 4° C will clear the antiserum, and the adsorbed serum will give only a very slight positive precipitation with normal mammary gland tissues without the agent but will still give a positive precipitation with tissues containing the agent.

The results of such an experiment are given in Table III-D, III-E and III-F. Comparing Table III-F with Table II-C, it is evident that practically all the antibodies against the normal tissue antigen, exclusive of the milk agent, had been removed from the adsorbed mouse mammary cancer antiserum. Table

TABLE III.

Precipitin Reactions of Adsorbed Serum Against Tissues with and without the Milk Agent.

D.

Serum dilutions	Antigen dilutions 1/60	1/120	1/240	1/480	1/960
1/6	+	+	+	+	—
1/12	+	+	+	+	—
1/24	+	+	+	+	—
1/48	+	+	+	—	—
1/96	+	+	+	—	—
1/192	+	+	—	—	—
1/384	+	+	+	—	—
1/768	+	+	—	—	—

Antigen: Mouse Mammary Cancer, 1/60 dilution = .016 mg N/cc.

Serum: Mouse Mammary Cancer Antiserum adsorbed with mammary gland tissue without milk agent.

E.

Serum dilutions	Antigen dilutions 1/60	1/120	1/240	1/480	1/960
1/6	+	+	+	+	—
1/12	+	+	+	—	—
1/24	+	+	+	—	—
1/48	+	+	+	—	—
1/96	+	+	—	—	—
1/192	+	+	+	—	—
1/384	+	+	+	—	—
1/768	+	—	—	—	—

Antigen: Mammary Gland Tissue with agent. 1/60 dilution = .007 mg N/cc.

Serum: Same as D.

F.

Serum dilutions	Antigen dilutions 1/60	1/120	1/240	1/480	1/960
1/6	+	±	—	—	—
1/12	+	—	—	—	—
1/24	—	—	—	—	—
1/48	—	—	—	—	—
1/96	—	—	—	—	—
1/192	—	—	—	—	—
1/384	—	—	—	—	—
1/768	—	—	—	—	—

Antigen: Mammary Gland Tissue without agent, 1/60 dilution = .007 mg N/cc.

Serum: Same as D.

III-D and Table III-E showed similar precipitation zone indicating that the removal of normal tissue antibodies did not remove the antibodies necessary to precipitate the common antigen or antigens found in mouse mammary cancer and normal mammary gland tissues containing the agent.

Summary. The data presented in this report describe a precipitin test for mouse tissues containing the milk agent. Antiserum produced in rabbits against mouse mammary cancer reacts in high titre with tissues containing the cancer agent and in low titre with tissues lacking the agent. Antiserum prepared against normal mammary gland tissues abundant with milk agent will precipitate mammary cancer antigen in high serum dilutions. Antiserum prepared against normal mammary gland tissues lacking the agent will precipitate mammary cancer antigen in low serum dilutions. Precipitin-adsorption reactions show that the removal of antibodies against mammary gland tissues without the agent in mouse mammary cancer antiserum will not remove antibodies which react with the common antigen or antigens found in mouse mammary cancer and mouse mammary gland tissues containing the milk agent.

The authors are indebted to Dr. J. J. Bittner of the University of Minnesota for generously supplying the mice and advising as to the strains of mice that should be used in this investigation.

16424

The Effect of Phrenicotomy on Gastrointestinal Mechanisms.*

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A number of observations point to a functional relationship between left diaphragm and stomach. Sauerbruch[1] has described a gastro-cardiac symptom complex after exeresis of the left phrenic nerve, consisting of cardio-vascular and gastro-intestinal disturbances, caused by changes in the gastro-intestinal tract. One factor was considered to be the pressure exerted by the stomach on the left cup of the diaphragm, resulting in displacement of the heart and large vessels. Bradycardia and premature systoles were ascribed to vagal stimulation, but occasionally, tachycardia and vertigo were observed. Frequently anxiety, epigastric distress, lack of appetite, pallor, nausea, vomiting and eructations occurred,[2] which were relieved by removing the subdiaphragmatic accumulation of air.[3] The latter did not seem to be due so much to swallowing of air, as to inability to belch it up. This symptom complex has been named "Roemheld's Syndrome."

Joannides[3] found that he could induce prompt contractions of the stomach by having the patient take deep breaths. This he ascribed to be due in part to the intermittent increase and decrease of intragastric pressure, induced by the contractions of the diaphragm. Joannides stated that cardiospasm may be an exaggeration of the normal contractions of the diaphragmatic pillars, and that partial contraction and relaxation of the diaphragm plays a role in belching.

Rickers[4] reported 2 cases of left side phrenic exeresis for tuberculosis, which developed the gastro-cardiac symptom complex.

* Aided by a grant from the Isaac and Kate Meyer Fund. The Department is in part supported by the Michael Reese Research Foundation.

1 Quoted by Roemheld, L., *Münch. Med. Wchnschr.*, 1928, **75**, 1872.

2 Noack, R., *Beitr. z. Klin. d. Tuberk.*, 1933, **82**, 397.

3 Joannides, M., *Arch. Int. Med.*, 1929, **44**, 856.

4 Rickers, L., *Beitr. z. Klin. d. Tuberk.*, 1933, **83**, 175.

X-ray studies revealed an elevation of the cupula of the diaphragm to the level of the fourth costal cartilage; the stomach extended perpendicularly downward, with a large gas bubble in the fundus. The pylorus was bent acutely to the right. Barium was found in the small intestine immediately after ingestion.

Noack[2] commented that the gastro-cardiac symptom complex after phrenic exeresis may be due in part to tearing and trauma of sympathetic fibers during the operative procedure. Reactivation of the diaphragm was observed 1½ to 2 years after phrenic exeresis and he assumed that, in addition to the phrenic nerves, the diaphragm was supplied by the 12th dorsal and by sympathetic nerves. In cases of left side phrenic exeresis the intestines were found to be pushed up, and a large gas bubble was seen in the stomach. The stomach was high, vertical, and had a rectangular kink at the pylorus, which was said to explain its rapid emptying. Food appeared to drop into the duodenum without staying in the stomach.

Gavazzeni[5] studied 2 patients before and after left side phrenic exeresis. Postoperatively, marked hypokinesis and decreased tonus of the stomach and a lack of gastric contractions after rapid respirations were observed. From these observations one could assume a slow rather than a rapid rate of emptying of the stomach.

Belbenoit[6] stated that complete and permanent cure of the gastro-cardiac symptom complex, if such should follow left side phrenic exeresis, may be obtained by pneumoperitoneum.

The nausea and vomiting present in cases of diaphragmatic hernia were decreased or abolished in a number of instances by left side phrenicotomy.[7–11] This probably has little to do with the gastro-cardiac symptom complex, and may be due mainly to afferent reflexes from diaphragm and stomach.

It is seen that the reports on left side phrenicotomy in relation to the stomach lack controlled data, and that most of the observations were complicated by such diseases as pulmonary tuberculosis or hiatus hernia. No adequately controlled work in normal human or animals was found.

Experiments. The effect of left side phrenicotomy was studied in the dog. In normal healthy animals, gastric emptying time was estimated according to the technique of Kozoll and Necheles.[12] Dogs, kept on a standard diet, were starved for 24 hours before fluoroscopy. Then they were fed a mixture of one pound of commercial dog food, mixed well with 70 g of barium sulfate and 150 cc water. They were fluoroscoped 15 minutes after feeding and thereafter every hour, until complete emptying of the stomach. Fluoroscopies were done at weekly and bi-weekly intervals. Left side phrenicotomy or vagotomy was performed aseptically under sodium pentobarbital anesthesia by the supradiaphragmatic approach. Following surgery, fluoroscopies were performed at weekly intervals as soon as the animals had recovered (5-6 days).

Results. In 4 dogs, following left side phrenicotomy, gastric emptying time decreased uniformly from an average of 6.3 hours to 4.3 hours. Fluoroscopically, gastric dilatation and hypotonia was observed and, after feeding, a relatively large gas bubble was seen

TABLE I.
Gastric Emptying Time in Hours.

Dog No.	Normal controls		Following left phrenicotomy	
	variation	Avg	variation	Avg
1	8,6	7	4,3,3,2	3
2	6,6,5	5.6	5,4,5,3,6	4.6
3	6,7,7,6,7,7	6.6	6,5,5,5,5,4	5.0
4	5,6,6,6,6,7	6.0	6,3,3,5,5,3	4.1

5 Gavazenni, M., *Il Policlinico* (sezione practica), 1935, **42**, 1343.

6 Belbenoit, S., *La Presse Méd.*, 1945, **53**, 88.

7 Eisen, D., *Can. Med. Assn. J.*, 1938, **39**, 207.

8 Ohler, W. R., and Ritvo, M., *New Eng. J. Med.*, 1943, **229**, 191.

9 Trueman, K. R., *Can. Med. Assn. J.*, 1947, **56**, 149.

10 Harrington, S. W., *J.A.M.A.*, 1933, **101**, 987.

11 Harrington, S. W., *West J. Surg., Obst., Gyne.*, 1936, **44**, 255.

12 Kozoll, D. D., and Necheles, H., *Surg.*, 1942, **2**, 360.

regularly in the fundus of the stomach. Yet, the barium emptied rapidly and was puddled in a moderately contracted small intestine. (Table I).

Since phrenicotomy shortened gastric emptying time considerably, its effect on the gastric retention following double vagotomy was tested. In 2 control dogs, following bilateral supradiaphragmatic vagotomy at the level of the hilus, gastric and esophageal atony with marked gaseous distention of stomach and intestine was observed. These dogs had a 24-hour gastric retention for 4 and 5 weeks respectively, during which time they vomited or regurgitated food and frothy mucoid gastric juice frequently, often intermittently, for periods of hours.

In 2 other dogs, following simultaneous bilateral supradiaphragmatic vagotomy and left side phrenicotomy, gastric atony, distention, and a large gas bubble in the gastric fundus were noted. However, no vomiting occurred, and little or no gas was observed in the stomach or intestine. The stomach appeared sausage shaped. A 24-hour gastric retention persisted for 3 weeks in both dogs. The animals did not appear sick, in contrast to the 2 dogs with vagotomy only. The striking differences between the above 2 groups of animals are demonstrated in Fig. 1 and 2. The x-ray films were taken 6 hours after feeding and 25 days after operation.

Discussion. A gastro-cardiac symptom complex following phrenic exeresis has been described by earlier authors. However, the gastro-intestinal symptoms of this complex, namely eructations, nausea, and vomiting were not noted by us in the dog. The gastro-cardiac symptom complex of phrenic exeresis in the neck may well be due to trauma to and avulsion of sympathetic fibers when the roots of the left phrenic nerve are pulled at or torn.

It has been explained by Noack[2] that the rapid emptying time following interruption

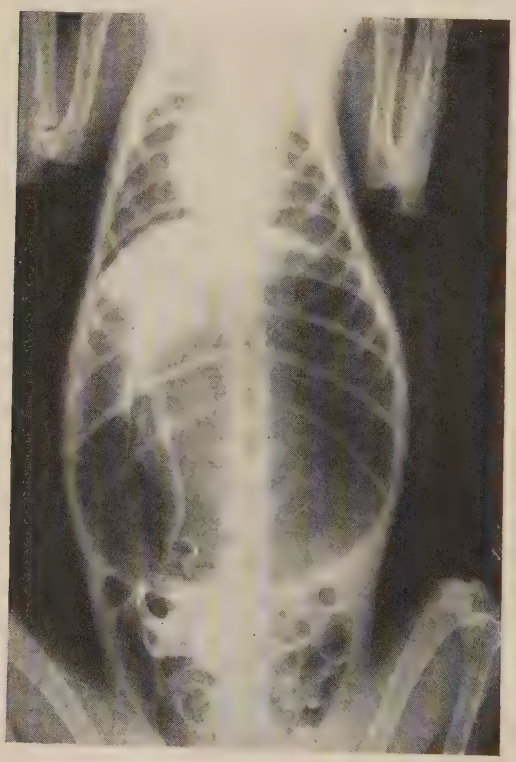

FIG. 1.
Bilateral supradiaphragmatic vagotomy, 25 days after vagotomy, 6 hours after feeding. Anterior-posterior view.

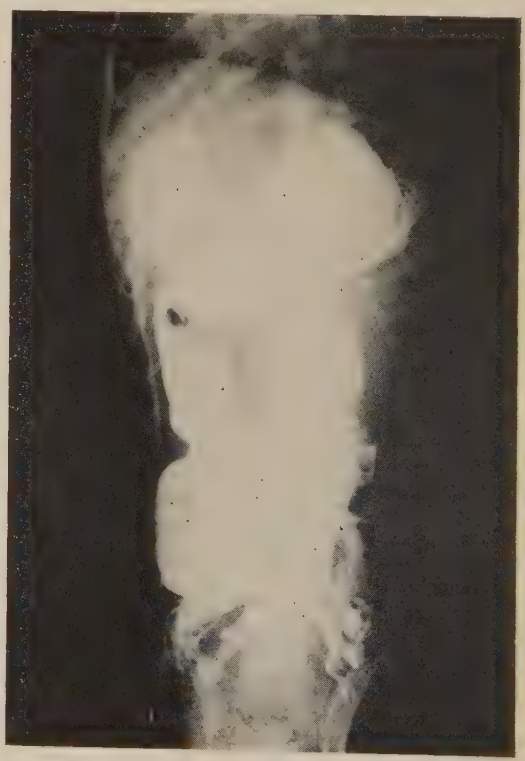

FIG. 2.
Bilateral supradiaphragmatic vagotomy plus left phrenicotomy, 25 days after operation, 6 hours after feeding. Anterior-posterior view.

of the left phrenic nerve may be associated with the mechanical elevation of the stomach, which leads to dumping of the food into the duodenum. Surprisingly, a similar rapidity of gastric emptying is seen in the dog, which does not assume the up-right position of the human. After phrenicotomy the stomach in the dog was ballooned, and hypotonia and hypokinesia were apparent, but there was never any vomiting. The small intestine appeared moderately contracted, with puddling of barium. Although a relatively large gas bubble was present in the fundus of the stomach after phrenicotomy, no or very little gas appeared in the intestine.

Following bilateral supradiaphragmatic vagotomy, there was much gas in the stomach and intestines and severe vomiting of frothy mucoid material was present, and the dogs appeared to be quite ill.

When the left phrenic nerve and both vagi were cut at the same time, there was no vomiting and the animals did not appear to be ill at any time postoperatively.

While left side phrenicotomy, performed in addition to bilateral vagotomy, does not relieve the retention and loss of tone of the stomach which follows bilateral supradiaphragmatic vagotomy only, yet, a number of disturbing complications of vagotomy such as gaseous distention and vomiting were not observed. It is possible that in case of atony of the stomach the pumping action of the left diaphragm forces air into the stomach, which cannot escape through the cardia, and part of which enters the intestine. Achalasia of the cardia may be present following supradiaphragmatic vagotomy and may be a factor in the retention of air in the stomach. Paralysis of the left diaphragm by phrenicotomy may abolish the pumping of air into the stomach and, by stretching the stomach forward, may facilitate escape of air through the esophagus.

Discrepancies in the clinical reports on gastro-intestinal effects of interruption of the left phrenic nerve may be due in part to the fact that the patients were suffering from advanced tuberculosis of the lungs, possibly also from intestinal tuberculosis. Furthermore, the method of interruption of the phrenic nerve is important, whether it was crushed, cut, or avulsed. In cases of avulsion or exeresis in the neck, sympathetic fibers may have been disturbed, thereby accounting for additional symptoms.

We do not propose left side phrenicotomy in cases of gastric atony and retention, but merely want to bring to attention some of the relations between the diaphragm and the gastro-intestinal system.

Summary. Section of the left phrenic nerve is followed by gastric hypotonia and hypokinesia and the presence of a large gas bubble in the fundus of the stomach. Gastric emptying time is shortened considerably. The barium appears to puddle in a moderately contracted intestine. There were no symptoms observed which could be associated with the so-called gastro-cardiac symptom complex. When phrenicotomy is done simultaneously with vagotomy, the vomiting and the gaseous distention of the gastro-intestional tract associated with bilateral supradiaphragmatic vagotomy only, are not seen.

16425 P

Relation Between Resting and Action Potential in the Frog Eye.*

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It has been observed that the magnitude (b-wave) of the action potential elicited from the excised frog eyeball in response to a light stimulus varies with the magnitude of the resting potential,[1] other conditions remaining

* This investigation was supported by a grant from the Research Board of the University of Illinois.

[1] Therman, P. O., *Acta Societatis Fennicae, N.S.B.*, 1938, **2**, No. 1.

constant. It has further been reported[2] that the above direct relation was consistently observed in the dark-adapted eyes of intact curarized frogs.

These observations imply that the sensitivity of the retina, measured by the retinal electric response to a constant intensity, constant duration light stimulus, is determined by the resting potential as well as by the state of adaptation.[3] The theoretical importance of this implication indicates the necessity for quantitative data.

Method. Experiments were performed on two types of frog eye preparations: (1) excised but intact eyeballs of frogs (*Rana pipiens*) and (2) excised enucleated eyeballs. Electrical contact with the eyes was made through agar impregnated wick electrodes connected to calomel half cells. Resting potentials were measured with a type K potentiometer; action potentials were recorded with an Offner condenser-coupled amplifier and crystograph. All animals used were dark-adapted 24 hours or longer and preparations were made under a weak red light. Constant intensity (expressed as foot candles, hereafter f.c.), constant duration (0.02 sec) test flashes were admitted at periodic intervals. Temperature varied in the range 23 to 25° C.

Results. 1. Experiments on excised intact eyeballs.

The results obtained varied with the intensity of the test flash. The protocols of typical experiments appear in Columns 1, 2 and 3, Table I. For low flash intensity (0.28 to 2.8 f.c.) the results are reasonably uniform and the ratio, $\frac{\text{action potential (b-wave)}}{\text{resting potential}}$ is fairly constant. For flash intensity of 28 f.c. the ratio is initially high and decreases during the experiment.

2. Experiments on excised enucleated eyes.

A protocol of a typical experiment appears in column 4, Table I. In general the action potential decreases as the resting potential

TABLE I.

Protocols from Experiments on Excised Intact Eyeballs, Col. 1, 2, and 3, and Excised Enucleated Eyeballs, Col. 4.

	Intact excised eyeballs of the frog.									Enucleated		
	Flash int. = 0.28 f.c.			Flash int. = 2.8 f.c.			Flash int. = 28 f.c.			Flash int. = 2.8 f.c.		
Elapsed time min.	Resting potential millivolts	b-wave magnitude millivolts	Ratio	Resting potential millivolts	b wave magnitude millivolts	Ratio	Resting potential millivolts	b-wave magnitude millivolts	Ratio	Resting potential millivolts	b-wave magnitude millivolts	Ratio
0	5.560	.288	.051	5.950	.270	.045	6.300	.400	.063	6.795	.495	.073
15	4.340	.168	.039	3.508	.100	.029	4.662	.214	.046	4.951	.442	.089
30	3.419	.168	.049	1.390	.076	.055	3.223	.106	.033	3.722	.410	.110
45	2.503	.140	.056	1.594	.079	.05	2.246	.052	.023	—	—	—
60	1.863	.122	.066	1.335	.066	.05	1.464	.039	.026	3.142	.417	.133
75	—	—	—	—	—	—	—	—	—	2.715	.362	.133
90	—	—	—	—	—	—	—	—	—	2.473	.350	.142

[2] Brossa, A., and Kohlrausch, A., *Arch. Anat. Physiol.*, 1913, 449.

[3] Riggs, L. A., *J. Cell. and Comp. Physiol.*, 1937, **9**, 491.

decreases, but not in a relatively uniform manner. The ratio, $\frac{\text{action potential}}{\text{resting potential}}$, typically increases during the experiment.

Discussion. The parallel variations in magnitude of the resting and action potentials of the frog eye are in agreement with the observations of Brossa and Kohlrausch. These investigators measured resting and action potentials with the same electrodes and with the same recording device, a string galvanometer.

Under these conditions changes in contact resistance might conceivably affect both resting and action potential in the same manner. In the experiments reported here the same electrodes were used but with two types of recording instruments: a null-point type K potentiometer and a condenser-coupled high-gain amplifier. Changes in contact resistance would affect the amplified reading much more than the potentiometer reading. Therefore, the parallel variations in resting and action potential must be attributed to the photoreceptor.

The data obtained from excised intact eyeballs are rather uniform for flash intensities of 2.8 f.c. or lower. For higher flash intensities the magnitude of the action potential drops off more rapidly than does the resting potential, indicating inadequate recovery of sensitivity between test flashes. The data from the excised enucleated eyeballs indicate consistently action potentials whose magnitudes decrease much more slowly than do the resting potentials. This difference between intact and enucleated excised eyes may possibly be due to differences in retinal area illuminated.

In conclusion, the action potential obtained from intact and enucleated excised frog eyes in response to constant intensity, constant duration light flashes varies as the resting or demarcation potential. The electrical response of the excised eye to light is determined by the state of dark-adaptation and by the magnitude of the resting potential.

16426

Fat and Nitrogen Absorption after Folic Acid Administration in Dogs Deprived of External Pancreatic Secretion.

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Interest in folic acid was aroused in this laboratory because of the similarity of the steatorrhea in sprue and in obstruction of the pancreatic ducts. Spies[1–5] and co-workers have contributed most notably to the advancing knowledge of folic acid. We wished to determine whether or not absorption of fat and nitrogen was influenced in pancreatic steatorrhea by the use of folic acid. In earlier work Spies *et al.*[5] mentioned that changes in the size and consistency of the stools in tropical sprue occur after treatment with folic acid, in that the stools became less bulky and more solid.

Darby and others[6] in a report of sprue cases from the Vanderbilt University Hospital state that stool examinations in 2 cases 2 months after treatment was begun still showed an increased fat content though diarrhea had

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1 Spies, T. D., *Lancet*, 1946, **1**, 225.

2 Spies, T. D., *J.A.M.A.*, 1946, **130**, 474.

3 Spies, T. D., Lopez, G. G., Menendez, J. A., Minnich, V., and Koch, M. B., *South. M. J.*, 1946, **39**, 30.

4 Spies, T. D., Vilter, C. F., Koch, M. B., and Caldwall, M. H., *South. M. J.*, 1945, **38**, 707.

5 Spies, T. D., Milanea, F., Menendez, A., Koch, M. B., and Minnich, V., *J. Lab. and Clin. Med.*, 1946, **31**, 227.

6 Darby, W. J., Jones, H. C., *J.A.M.A.*, 1946, **130**, 780.

ceased. Their patients had received daily 15 mg of folic acid given intramuscularly. Fat determinations in their cases showed that prior to treatment in one case 37% of the dry weight of one stool specimen and 44% of a second dried stool were composed of fat. After treatment the fat fell to 29% of the weight of the dried stool. In another of their cases fat comprised 40% of the dry weight of a stool before treatment and a third case 23%. Stool analyses after treatment in these last 2 cases were not given. Fecal nitrogen was also determined in their 3 cases prior to treatment. The daily amount was 3.91 and 4.89 g in Case 1; 2.9 g in Case 2; and 4.11 g in Case 3. No figures were given for similar studies after treatment.

A report by Davidson *et al.*[7] indicates that folic acid did not improve fat-absorption, as estimated by fat balance tests, in 5 out of 6 cases of the sprue syndrome.

Some writers report a smaller percentage of fat in the stools after various forms of treatment in cases of sprue and pancreatic disease. The percentage of fat alone may give a false impression of the true state of affairs. It may fall simply because the patient is eating less fat. The only way to judge accurately increased absorption of fat resulting from treatment is to have the patient on a standard diet before, during, and after the prescribed treatment. It has been the practice of many investigators to express the fat content of the stools simply as percentage of dried matter. More important is the daily quantity of fat excreted in the stools. To determine the amount of fat absorbed it is necessary in addition to know the exact amount of fat in the diet; hence the importance of a standard diet in which the amount of fat and nitrogen has been determined.

Methods. Absorption experiments were carried out on 5 dogs deprived of external pancreatic secretion. Avoidance of complete pancreatectomy made it unnecessary for us to give our dogs insulin as they did not develop diabetes. The pancreatic juice was

[7] Davidson, L. S. P., Girdwood, R. H., and Innes, E. M., *Lancet*, 1947, **1**, 511.

TABLE I.

Folic Acid Added to Standard Diet of Five Dogs Deprived of Pancreatic Secretion.

Dog	I	I	I	I	I	II	III	IV	V
Date 1947	Mar. 24-27	Mar. 31-Apr. 2	Apr. 18-21	Apr. 25-28	May 2-5	May 9-12	June 19-22	June 19-22	June 19-22
Folic acid daily—mg	0	20	50	50	100	200	50	50	50
Wet wt feces—g	317	300	150	272	288	377	119	352	224	234
Dry " "	87	—	39	87	86	104	38	110	79	71
Percent solids	27.4	—	26.1	31.9	29.8	27.5	32.4	31.2	36.9	30.4
Nitrogen fed—g	14.6	12.9	9.2	14.6	14.6	14.6	10.7	12.9	12.9	12.3
" excr.—g	5.7	7.8	2.4	4.9	6.3	7.0	2.7	5.6	5.5	5.1
% N absorbed	60.6	72.7	75.2	66.5	56.5	52.2	74.8	56.7	57.1	61.8
Fat fed—g	26.8	25.1	21.6	26.8	26.8	26.8	18.9	23.5	20.7	20.2
" excr.—g	12.9	15.8	7.0	15.6	12.2	14.5	7.9	18.2	11.5	12.5
% fat absorbed	52.0	36.9	67.8	41.9	54.5	46.0	58.4	22.8	44.5	38.3

Notes: Weight of feces and food indicate the average daily weight. All experiments were carried out over three-day periods.

excluded from the intestine by ligating all the pancreatic ducts and interposing the omentum between the pancreas and duodenum. The pancreas of the dog frequently has more than 2 ducts so that care must be exercised in carrying out the procedure. A balanced diet containing known quantities of the various food components was fed and additions of 20, 50, 100, and 200 mg of folic acid were made to the diet.

In these experiments the dogs were fed diets composed of ground horse meat, evaporated milk and a commercial dog food, in some instances Gaines Meal, manufactured by General Foods Corporation and in other instances Kibble Dog Food, manufactured by the Wilson Company. Figures in Table I indicate that varying quantities of fat were fed in different experiments. Actually these figures represent the amount of food which the dogs ate. They were offered a diet on which normal animals in our laboratory gained weight and which was adequate in all components.

To mark the stools carmine was given at the beginning and charcoal at the end of 3-day feeding experiments. All the stools were collected and analyzed quantitatively for total solids, fat, and nitrogen. Fractional determinations were made for neutral fat, fatty acids, and soaps. The animals were later examined at autopsy in order to obtain evidence of definite occlusion of the pancreatic ducts and the presence or absence of aberrant pancreatic tissue.

Results. These are shown in Table I. Five experiments were performed on dog I giving 20 to 200 mg of folic acid daily. Two hundred mg of folic acid are far in excess of the usual amounts required in the treatment of sprue. In the control experiment when no folic acid was given, 52% of the fat fed was absorbed. After folic acid was added to the diet, the average percentage of fat absorbed was 50. There was not only no correlation between the fat absorption and the quantity of folic acid administered, but no increase in absorption even when a very large amount of folic acid was given.

Studies were made on 4 additional dogs deprived of pancreatic digestion. They were given 50 mg of folic acid daily, an amount well above the usual therapeutic dose of folic acid used in gastrointestinal diseases. The percentage of fat fed that was absorbed ranged from 22.8 to 58.4; the average being 41%. The evidence is conclusive that folic acid did not prevent great loss of fat in the stools.

At autopsy of the dogs no evidence was found of any connection between the atrophic pancreatic remains and the duodenum, and there was no aberrant pancreas.

The stools were larger than those of normal animals, yet the percentage of solids varied from 26.1% to 36.9%, which is within the normal range. Nitrogen absorption during the period of folic acid administration varied from 52.2% to 75.2%.

Determinations of the components of the total fecal fat, while folic acid was given, resulted in figures for neutral fat, fatty acids, and soaps which are no different from the results obtained in stool examinations previously made in other studies in our laboratory on animals deprived of the external secretion of the pancreas.

From the results obtained there is no evidence that folic acid given to animals with steatorrhea due to absence of pancreatic digestion will further the absorption of fat or nitrogen.

As normal stools contain a certain quantity of fat, even in starvation, sufficient quantities of fat must be given to produce an amount definitely in excess of the normal figure of intestinal "fat secretion." Normally, fecal fat is considered to be largely endogenous. Previous work with animals in our laboratory indicates that this secretory figure lies somewhere between 0.5 and 2.0 g daily. The figures of fat lost in the feces as given in the table are greatly in excess of this amount. In earlier experiments on one of the animals we observed marked variations in absorption of food components. It first appeared that improved absorption was due to the folic acid, but later found to be only the usual variation in absorption which occurs in animals deprived of pancreatic digestion.

TABLE II.
Chart Showing the Difference Between Percentage of Fat in Stools and Percentage of Fat Excreted.

Dog Date	VI 12/20/46	VI 1/8/47	I 12/20/46	I 1/8/47	VII 12/23/46	VII 1/8/47
Wt dry stool (g)	107	65.9	113	110	55	28
Total (g) fat excreted	18.3	19.9	14.9	21.6	4.6	3.5
% dry weight	17.1	30.2	13.2	19.7	8.4	12.6
Fat fed (g)	38.1	38.1	38.1	38.1	38.1	38.1
% fat excreted	48.1	52.3	39.2	56.8	12.2	9.2
	Dogs deprived of external pancreatic secretion				Normal dog	

Weights indicate average daily weight. All experiments were carried out over 3-day periods.

The percentage of fat in the dried stool in normal dogs on a standard diet varies widely. In healthy human beings on the Schmidt diet, the average in our clinic has been 25%. In steatorrhea of pancreatic origin the percentage of fat in the dried stool is frequently less than the maximum occurring in health; hence no definite conclusion regarding the absorption of fat can be drawn from the percentage of fat in the stool unless it is greatly elevated. In Table II are given the results of absorption studies on 2 dogs deprived of pancreatic secretion, and for comparison those on a normal dog. All were fed the same amount of fat daily, namely 38 g, and all had a percentage of fat in the dried stools that was within normal limits, yet the dogs without pancreatic digestion lost in their feces from 39.2% to 56.8% of the fat fed. It is evident from this that the amount of fat in the stools is all important. In obstruction of the pancreatic ducts the weight of the dried stools is definitely increased as is shown in Table II.

Summary. Data on the absorption of fat and nitrogen following folic acid therapy in dogs deprived of external pancreatic secretion have been presented. Folic acid was shown to be of no value as far as absorption of these substances was concerned.

The importance of employing a standard diet of known composition and of determining the amount of fat lost in the stools and not relying on the percentage of fat present in the dried stool is emphasized.

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Blocking and Protecting Actions of Amines and Ammonium Compounds on a Crustacean Synapse.

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The central nervous system of the crayfish provides a highly satisfactory preparation for the study of synaptic transmission. It contains 4 giant fibers, up to 250μ in diameter, which extend the entire length of the ventral nerve cord. These fibers synapse with motor fibers in each of the abdominal ganglia. A single impulse in a giant fiber normally causes a single response in the motor fibers (Wiersma[1]).

Nicotine is a particularly active drug on this synapse, causing an initial facilitation followed by block. However, approximately 40 minutes after block by nicotine, transmis-

[1] Wiersma, C. A. G., *J. Neurophysiol.*, 1947, **10**, 23.

sion re-appears, regardless of whether the preparation has been rinsed with fresh perfusion fluid or allowed to remain in the nicotine solution. In either case, preparations which have recovered from nicotine block are protected against further action of nicotine, since renewed application of this drug, in the same or even in higher concentration, is without effect (Wiersma and Schallek[2]; Schallek and Wiersma[3]).

These authors also found that high concentrations of nornicotine and anabasine, 2 compounds closely related in structure to nicotine, block transmission across this synapse. Lower concentrations have no blocking action, but they are able to protect the synapse against the effects of a subsequent application of nicotine.

In amphibia and mammals nicotine shows both stimulatory and inhibitory effects on ganglionic and central synapses of the autonomic nervous system as well as at the peripheral neuro-muscular junctions of the somatic nervous system. The actions of the aliphatic primary amines and trimethylammonium salts have been found to be similar to those of nicotine upon various mammalian test preparations (Alles[4]). The present study describes the actions of amines and trimethylammonium salts on synaptic transmission in the crayfish. Observations were made on both the direct action of these compounds and on their ability to protect the preparation against the action of nicotine.

Methods. The procedure used was based on that described by Wiersma and Schallek[2] and Schallek and Wiersma.[3] The abdominal nerve cord of the crayfish *Cambarus clarkii* was freed from surrounding tissue; the preparation was then placed in a bath containing 100 cc of crayfish perfusion fluid (van Harreveld[5]). Stimulating electrodes were placed on the nerve cord between the 5th and 6th abdominal ganglia, while the response, which was led off a motor root of the 2nd or 3rd ganglion, was observed on the screen of a cathode-ray oscilloscope. Stimuli were delivered at a rate of 30 per minute. Control tests showed that in the absence of drugs the post-ganglionic response to this rate of stimulation remained unchanged for several hours. Drug concentrations mentioned in this paper are final values after dilution in the bath.

In testing amines and ammonium compounds, their direct action was noted by finding the minimum concentration causing block within 10 minutes. To avoid possible osmotic effects, concentrations higher than 5×10^{-3} g/cc were not used. After finding the minimum blocking concentration, lower concentrations of the drug were tested for their protection against nicotine. Control tests on other preparations had shown that in the absence of other drugs the nicotine dose used (1 or 2×10^{-5}) blocked transmission in 2 to 5 minutes. The nicotine was always added to the bath 10 minutes after application of the compound being studied. If synaptic transmission still occurred 10 minutes later, the synapse was considered to be protected against the blocking action of nicotine. If nicotine block occurred in 5-10 minutes, the concentration of the drug being tested was considered to show a trace of protection against nicotine.

This procedure is illustrated by the following experiments on heptyl amine sulfate:

A. 1×10^{-3} g/cc blocks in 1 min. Hence this dose is above threshold for block.

B. 5×10^{-4} blocks transmission in 10 min. This concentration is the threshold for block as defined in this study.

C. 1×10^{-4} has no effect in 10 min. Nicotine 1×10^{-5}, given at this time, produced only a transient depression. Hence this concentration of heptyl amine is below threshold for block, but above threshold for protection against nicotine.

D. 5×10^{-5} has no effect in 10 min. Nicotine 1×10^{-5} now blocks in 8 min. Hence this is approximately the threshold dose for protection against nicotine.

2 Wiersma, C. A. G., and Schallek, W., *J. Neurophysiol.*, 1947, **10**, 323; *Science*, 1947, **106**, 421.

3 Schallek, W., and Wiersma, C. A. G., *J. Cell. Comp. Physiol.*, 1948, **31**, 35.

4 Alles, G. A., *Univ. Calif. Publ. Pharmacol.*, 1941, **2**, 1.

5 Harreveld, A. van, PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 428.

TABLE I.
Action of Amine Compounds.
Minimum bath concentrations in g/cc for action indicated.
Concentrations higher than 5×10^{-3} were not used.

Compound	Block	Protection against 1×10^{-5} nicotine
Butyl amine sulfate	None	None
Amyl " "	"	5×10^{-4}
Hexyl " "	5×10^{-3}	5×10^{-4}
Heptyl " "	5×10^{-4}	5×10^{-5}
Octyl " "	5×10^{-5}	1×10^{-5}
Dodecyl " HCl	1×10^{-5}	2×10^{-6} (trace)
Butyl methylamine HCl	None	None
Amyl " "	1×10^{-3}	1×10^{-4}
Hexyl " "	1×10^{-3}	1×10^{-5}
Heptyl " "	1×10^{-4}	1×10^{-5}
Dodecyl " HBr	5×10^{-5}	5×10^{-6} (trace)
Butyl dimethylamine HCl	1×10^{-3}	1×10^{-4}
Amyl " "	1×10^{-3}	1×10^{-4}
Hexyl " "	5×10^{-4}	1×10^{-4}
Heptyl " "	2×10^{-4}	1×10^{-4}
Octyl " "	1×10^{-4}	5×10^{-6}
Dodecyl " "	5×10^{-5}	5×10^{-7} (trace)

E. 1×10^{-5} has no effect in 10 min. Nicotine 1×10^{-5} now blocks in 4 min. Hence this concentration shows no protection against nicotine.

It is obvious that a great many experiments would be needed for exact quantitative determinations of the actions of each compound. The threshold values presented in this study should be considered only as good approximations.

Observations. Amine Compounds of Table I. Each of 3 series of alkyl amines shows a variable but progressive increase in blocking activity as the alkyl chain is lengthened. With the lower alkyl compounds the dimethylamines are generally more active in this respect than the corresponding methylamines and they in turn more active than the amines. Dodecyl amine, however, appears to be more active than either its methyl or dimethyl derivatives.

The ability of these alkyl compounds to protect the synapse against blocking by nicotine also shows a general increase with the length of the alkyl chain through the octyl compounds. The dodecyl amines, however, show only partial protection against subsequent nicotine blocking, although it is notable that this partial protection is shown by high dilutions. It is possible that the protection which might be expected to be produced by the dodecyl compounds is largely obscured by their powerful direct blocking action. Evidence for this view is presented below.

Trimethylammonium Compounds of Table II. The methyl to octyl trimethylammonium salts show no blocking activity in the highest concentrations tested (5×10^{-3} g/cc). The dodecyl compound was quite active in this respect, but less so than the other dodecyl compounds.

The ability of these alkyl trimethylammoniums to protect the synapse against blocking by nicotine shows a general increase with the length of the alkyl chain. While the lower members of the series show less protecting action against the effect of nicotine than the corresponding amine compounds, the dodecyl compound is quite comparable in effect.

The amyl trimethylammonium salt was notable in being the only substance found in this study, aside from nicotine itself, that caused a consistent facilitation of synaptic transmission. A weak facilitation was occasionally noted with the closely related butyl and hexyl trimethylammonium salts. It should be noted that observations of facilitation are far more variable and dependent upon the state of the particular preparation than

TABLE II.
Action of Trimethyl Ammonium Compounds.
Minimum bath concentrations in g/cc for action indicated.
Concentrations higher than 5×10^{-3} were not used.

Compound			Block	Protection against 1×10^{-5} nicotine
Methyl	trimethylammonium	I	None	—
Ethyl	,,	,,	,,	—
Propyl	,,	,,	,,	—
Butyl	,,	,,	,,	None
Amyl	,,	,,	,, *	5×10^{-3}
Hexyl	,,	,,	,,	2×10^{-4}
Heptyl	,,	,,	,,	5×10^{-4}
Octyl	,,	,,	,,	5×10^{-5}
Dodecyl	,,	Br	5×10^{-4}	5×10^{-7} (trace)

* Shows facilitation at 1×10^{-3}.

TABLE III.
Protection Against Nicotine.
Blocking time in minutes after 2×10^{-5} g/cc of nicotine given 10 minutes after concentrations of trimethylammonium iodides.

Conc. of trimethylammonium compound	Hexyl	Heptyl	Octyl	Dodecyl
5×10^{-3} g/cc	>60	7	6	—
1×10^{-3} ,,	26	20	16	—
5×10^{-4} ,,	12	5	>60	—
1×10^{-4} ,,	8	—	10	2
1×10^{-5} ,,	—	—	6	5
1×10^{-6} ,,	—	—	—	7
1×10^{-7} ,,	—	—	—	3

Nicotine controls, without protecting compounds, block in 2-5 minutes.

are observations of inhibition.

Dodecyl Compounds. It was suggested above that the failure of these compounds to show complete protection against the action of nicotine might be correlated with their powerful direct blocking activities. Evidence in support of this is given in Table III where the blocking time required by a test dose of nicotine is shown in relation to the concentration of the protecting compound used.

The hexyl compound, as well as all compounds below it in this series, shows maximum protection against the effect of nicotine at the highest concentration tested (5×10^{-3} g/cc). A different situation appears with the heptyl, octyl and dodecyl compounds. Here the highest concentration which does not block by itself in 10 minutes shows little or no protection against the test dose of nicotine. Maximum protection is afforded by pretreatment with a lower concentration of ammonium compound. It seems reasonable to assume that the decreased protection apparent with the higher concentration of these compounds is caused by a synergism between their own not fully developed blocking action and that of the added test dose of nicotine. This synergism may likewise account for the fact that the dodecyl compounds, while exerting the most powerful blocking actions of the series, show no more than a trace of protection.

Discussion. The present study is the first to compare the actions of primary, secondary and tertiary amines on the same preparation. Alles[4] studied the alkyl primary amines extensively on mammalian test preparations. He found that their inhibitory actions on gut of the guinea pig and rabbit, and their antagonism to the actions of adrenalin, acetylcholine and histamine, increased regularly with the length of the alkyl chain. These relationships appear to be the same as found in the present paper for the direct and indirect actions of each of the series of primary, secondary and tertiary amines.

With regard to the alkyl trimethylammoniums, two groupings of depressant effects

seem necessary. These are the curariform actions, usually studied with frog nerve-muscle preparations, and the muscarinic actions, usually studied with the frog heart or the mammalian circulatory system. Studies on the curariform action of a series of these salts showed that the amyl through octyl compounds were about equal in activity, while the dodecyl compound was much less active (Ing and Wright[6]). Studies on the muscarinic activities of the methyl to nonyl trimethylammonium salts clearly showed the butyl and amyl members of the series to be the most active (Kulz,[7] Raventos,[8] Alles and Knoefel[9]). In marked contrast to both these results, the dodecyl compound is the only one which shows any blocking activity on the crayfish synapse.

The stimulant or nicotinic actions of the alkyl trimethylammoniums have been studied on the leech muscle, frog rectus abdominis, gut of rat or rabbit, and circulatory system of the dog. The butyl, amyl or hexyl compounds were found to be the most active of the series (Kulz and Achenbach,[10] Raventos,[8] Alles and Knoefel[9]). Fitting in with these observations, we find in the present work that the amyl compound is noteworthy in showing a marked facilitation of the synapse studied, while the butyl and hexyl compounds occasionally show a weak facilitating action.

The antagonism of nicotine by quaternary ammonium compounds has not been studied previously. However, Raventos[8] and Clark and Raventos[11] investigated the antagonism of the nicotinic actions of acetylcholine by a series of alkyl trimethylammonium compounds. They found that the blocking activity of this series increased with the length of the alkyl side chain. In the present paper a similar relationship has been found for the blocking activity against the action of nicotine. It therefore appears that the blocking action of these compounds to nicotine on the crayfish synapse corresponds to their blocking action to acetylcholine on other preparations. This is remarkable, for no direct action of acetylcholine has been observed on synaptic transmission in the crayfish; neither facilitation, nor block, nor protection against nicotine (Prosser,[12] Schallek and Wiersma[3]).

Summary. This paper describes the effects of various amines and ammonium compounds on synaptic transmission in the central nervous system of the crayfish.

In the series from butyl to octyl amine, the activity of the drug in blocking synaptic transmission increases with the length of the alkyl side chain. Dodecyl amine shows greater activity than octyl amine. The corresponding alkyl methylamines and dimethylamines show a similar increase. The trimethylammonium series shows no blocking activity until the dodecyl compound is reached. The amyl trimethylammonium compound is unique in showing a consistent facilitation of synaptic transmission.

The ability of sub-threshold doses of these drugs to protect the preparation against the blocking action of nicotine reaches a maximum with the octyl compounds. The protecting activity of the trimethylammonium series is generally weaker than that of the other compounds. Although the dodecyl compounds show only a trace of protection against nicotine, this weak protection is shown at very high dilution.

[6] Ing, H. R., and Wright, W. M., *Proc. Roy. Soc.*, 1931, **109B**, 337.

[7] Külz, F., *Arch. Exp. Path. Pharmakol.*, 1923, **98**, 339.

[8] Raventos, J., *Quart. J. Exp. Physiol.*, 1937, **36**, 361 and **37**, 99.

[9] Alles, G. A., and Knoefel, P. K., *Univ. Calif. Publ. Pharmacol.*, 1939, **1**, 187.

[10] Külz, F., and Achenbach, W., *Arch. Exp. Path. Pharmakol.*, 1923, **100**, 61.

[11] Clark, A. J., and Raventos, J., *Quart. J. Exp. Physiol.*, 1937, **37**, 375.

[12] Prosser, C. L., *J. Cell. Comp. Physiol.*, 1940, **16**, 25.

16428

Presence and Significance of Desoxyribose Nucleoprotein in the Purulent Pleural Exudates of Patients.*

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In an investigation which has consisted of determining the effect of injections of partially purified streptococcal fibrinolysin (streptokinase) on fibrinous and purulent exudations, detailed biochemical assays of the solid and fluid content of samples of the exudations have been made before and after the injections. In the course of the study evidence has been accumulated that there is present as a conspicuous part of the thick inflammatory exudates a considerable amount of a fibrous protein which is not fibrin or its denaturation products. The coarse characteristic coagulum and thick sediments of purulent material has been generally interpreted as being due to its cellular and fibrinous content. However, it is the purpose of this report to describe and define the presence in considerable amounts of a nucleoprotein as a constituent part of purulent exudations and to indicate its importance in accounting for such characteristics of pus as thickness, sliminess, viscosity, and stringiness.

In addition, in an accompanying article,[1] the elaboration by hemolytic streptococci of a product which rapidly liquefies the fibrous nucleoprotein will be described. This property of the organisms although different from the fibrinolysin (streptokinase) also produced by hemolytic streptococci, parallels the presence of the latter in an interesting although not inseparable manner.

The fact that the solid sediment of purulent empyemal material was not solely fibrin was first suggested by observations made on samples of pus derived from a case of empyema due to Friedlander bacillus infection. It was noted that when the pH of this sample was raised to above 10, by the addition of alkali, a major proportion of the sediment went into solution which now became diffusely viscid, and that when the solution was brought back to neutrality by the slow addition of 0.1 N HCl, a fibrous stringy material formed, which redissolved again in alkaline mediums. Additional procedures were performed as follows: 25 ml of a tenfold dilution of the same sample of pus was added to 100 ml of 95% alcohol, stirred, and allowed to stand overnight. The precipitate that formed was filtered, washed with alcohol, and dried with ether. The dried powder was insoluble in water, normal saline solution, and 0.1 N HCl but was readily soluble in 0.1 N NaOH, forming a viscid solution. This solution was found after acid hydrolysis to reduce Benedict's reagent. On the basis of the results just mentioned it was apparent that the characteristics were not those of fibrin. On the other hand the presence of a reducing sugar, the solubilities, and the stringy fibrous physical character led to an exploration of the possibility that the substance was nucleoprotein.

Isolation of Nucleoprotein from Purulent Exudates of Patients. The sediments from 2 cases of purulent empyema, based on mixed infections superimposed on tuberculosis, were analyzed in the following manner: The specimens, adjusted to neutral pH, were thoroughly washed with physiological saline, and then extracted with 1 M NaCl. The supernatant portion became viscid and opalescent. The addition of 6 volumes of distilled water to the NaCl extract resulted in the immediate ap-

* This study was supported by a grant from the National Institute of Health, United States Public Health Service.

† Supported by a grant to the Department of Microbiology, from the Life Insurance Medical Research Fund.

1 Tillett, W. S., Sherry, S., and Christensen, L. R., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 184.

pearance of a heavy fibrous precipitate, which redissolved in 1 M NaCl and reprecipitated in 0.14 M NaCl. Materials obtained after 6 reprecipitations from M NaCl extracts were shown to contain N, P, and to give a positive biuret reaction. After acid hydrolysis positive purine and Molisch tests were obtained.

A similar analysis yielding identical results has been carried out on 10 samples of pleural exudation derived from patients having pulmonary infections of different etiologies. In some instances causative bacteria, such as pneumococci, aerobic and anaerobic streptococci, and others, were present in the exudates and in other instances the specimens were sterile. It is apparent, therefore, that the material derived from the specimens was not a product of the organisms but resulted from the inflammatory reaction. Although not described in detail in this article, a substance with comparable chemical and physical characteristics has been derived from the buffy coat of human blood.

The above chemical findings which identify the substance from exudates as being a protein and containing purines, phosphorus, and reducing sugars serve to demonstrate its nucleoprotein characteristics. The physical characteristics of the nucleoprotein derived from the exudates is also of interest in connection with its significance in contributing to the total gross appearance of pus. The material which has been isolated is fibrous and stringy. Its nature and abundant yield from a purulent exudate, is shown in Fig. 1. When the pH of the solution was raised to 8-9 by the addition of 0.1 N NaOH, the fibrous portion became slimy, swollen and formed a coagulum. When the pH was raised above 10 the substance dissolved forming a viscid solution. Mirsky[2] refers to the earlier report of Hoppe-Seyler made in 1865 in which the author described the recovery of a fibrous material from pus cells but did not identify it further.

Estimations of Amount of Nucleoprotein in Samples of Purulent Exudations. All of the preliminary experiments have indicated

FIG. 1.
The fibrous stringy nucleoprotein derived from 25 g of purulent exudate is shown suspended in 3 liters of 0.1 M NaCl. In this instance the nucleoprotein comprised 70% of the dried solids and 50% of the nitrogen of the total sediment.

that the nucleoprotein increment of the exudates constituted a surprisingly large amount of the total sediment. Accordingly quantitative estimations were carried out on 3 specimens. Using the dry weight and N content of the total sediment and that of the isolated nucleoprotein, it was found that the latter comprised 30-70% of the total solids and contained 26-50% of the nitrogen. This finding takes on additional interest and importance in connection with the studies on the production of desoxyribonuclease by hemolytic streptococci,[1] and in our investigation on the effect of the injection of partially purified, nontoxic streptococcal concentrates into patients, a subject which will be reported in a subsequent communication.

Isolation of Nucleic Acid from Nucleoprotein Derived from Pus. Three different samples

[2] Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, 1947, **30**, 117.

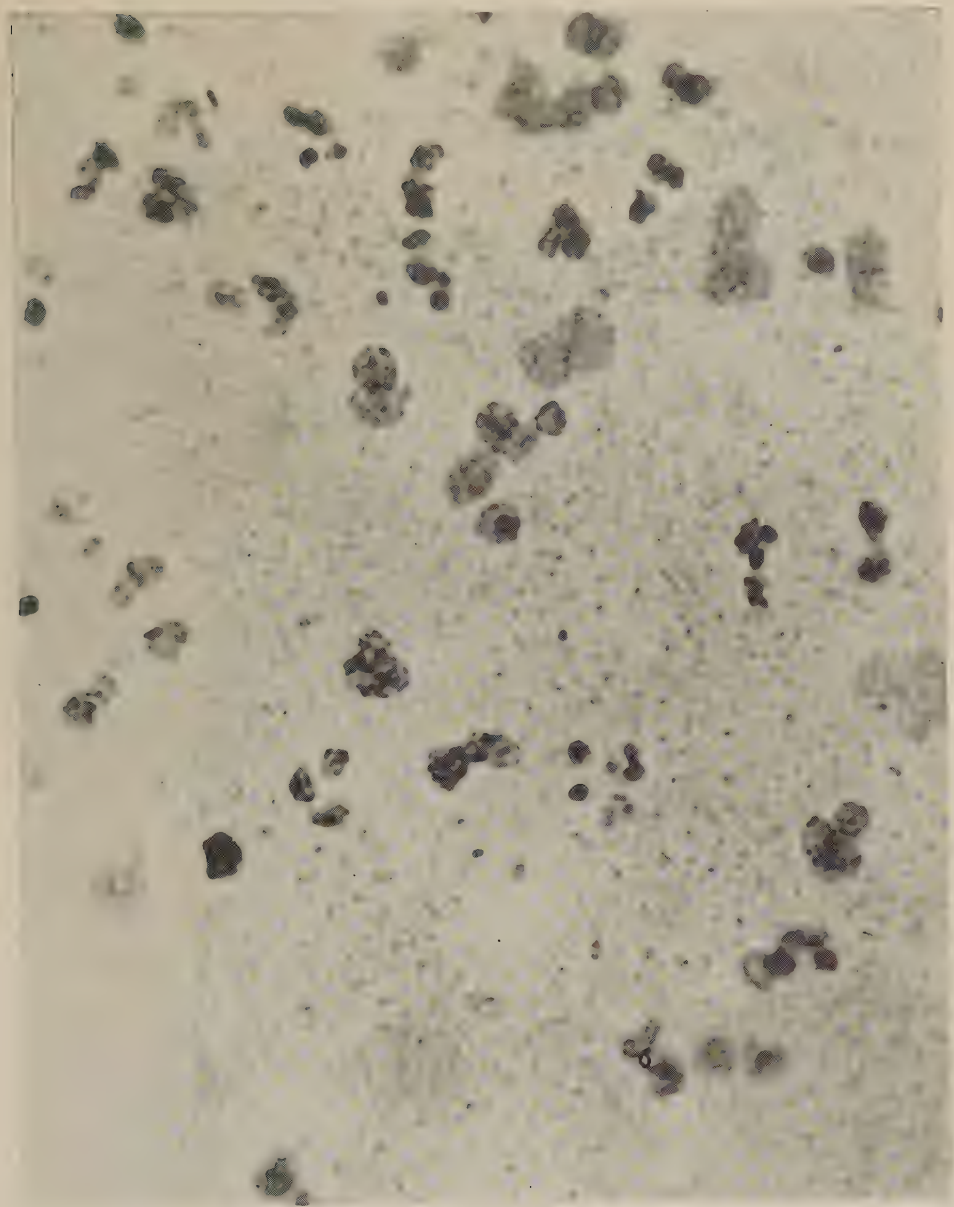


FIG. 2.

Specimen of early pleural exudate (sterile) from a patient with pneumonia. Stained by Feulgen method. 1766 × magnification. Nuclei of W.B.C., some collections of lattice work stroma of an undetermined nature, and large numbers of extracellular granules are stained.

of purulent sediment were extracted with 1 M NaCl after thorough washing with physiological saline. The viscid opalescent supernatant portions were centrifuged clear of debris, and the nucleoprotein precipitated by addition of six volumes of distilled water. After several reprecipitations, the precipitate was finally dissolved in alkalinized M NaCl, and protein removed by shaking with a chloroform-octyl alcohol mixture (4:1) followed by centrifugation. After about 8 such shakings, and when no more precipitate formed on 2

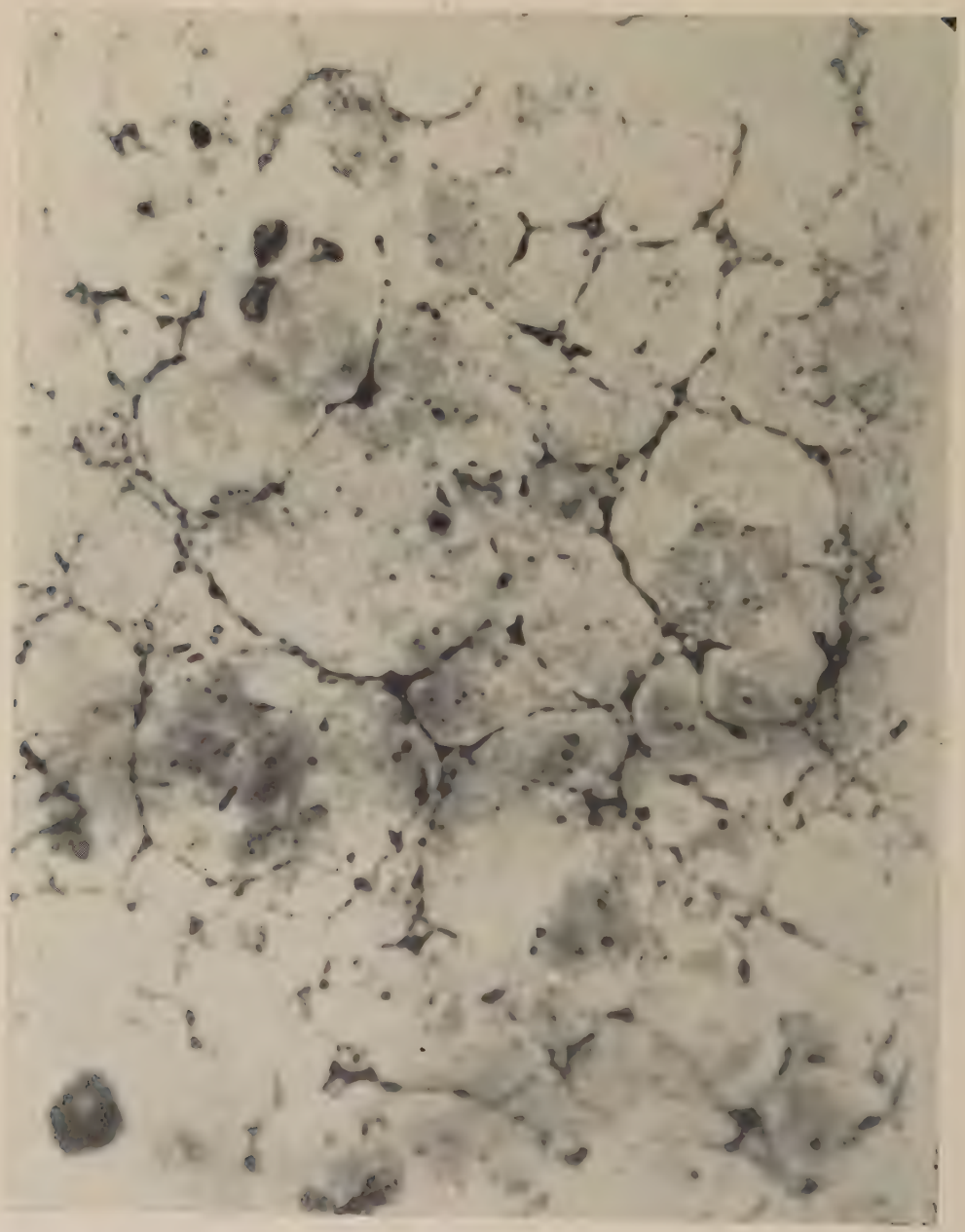
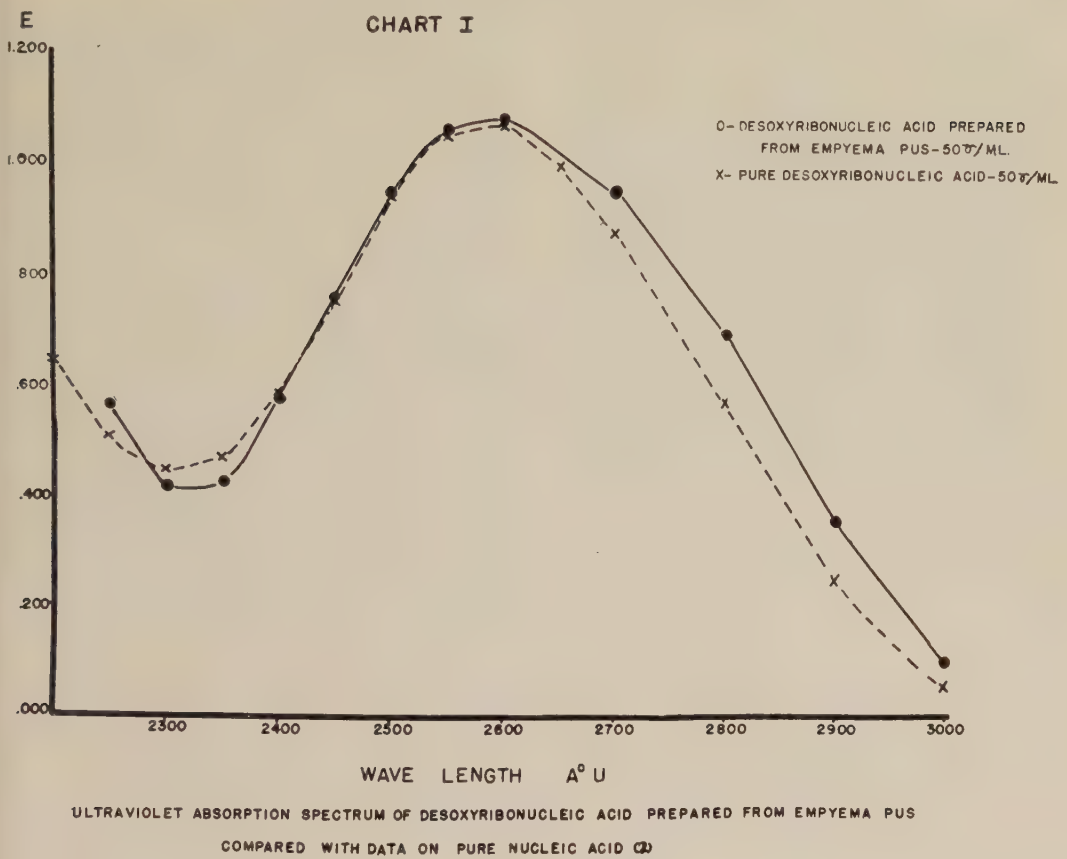


FIG. 3.

Specimen from protracted case of tuberculous empyema. Stained by the Feulgen method. 1765 × magnification. Only occasional leucocyte present. Extensive extracellular reticulum, together with some granules and areas of amorphous formation have taken up the stain.

successive treatments, the supernatant was neutralized and added to 2 vols. of alcohol. The precipitate that formed overnight was dried with ether. The powders obtained were water soluble and yielded very viscous solutions, which showed birefringence of flow indicating a high degree of polymerization. The absorption spectrum of one of the samples is shown in Fig. 2. Its absorption at 2600A in a concentration of 50 γ ml was



ULTRAVIOLET ABSORPTION SPECTRUM OF DESOXYRIBONUCLEIC ACID PREPARED FROM EMPYEMA PUS COMPARED WITH DATA ON PURE NUCLEIC ACID (2)

within 3% of that characteristic of pure nucleic acid.[2] The N content was 14.95%, P 8.1%, N/P ratio 1.84. It was biuret negative and gave a strongly positive Dische diphenylamine reaction.[3] Because of the methods of extraction that were successful, the results of the chemical analyses and the essential identical similarity of its properties to a highly purified preparation of sodium thymonucleate prepared by the Hammarsten method from calf thymus which was used as a control for comparison,‡ the materials were identified as a desoxyribose nucleic acid. The identity of the substance as desoxyribonucleic acid was further established by the demonstration of its depolymerization with both desoxyribonuclease and also with concentrates of hemolytic streptococcal filtrates containing desoxyribonuclease which are described in the subsequent article.[1]

For further observations on the presence of desoxyribose nucleoprotein in inflammatory exudates in large amounts, preparations have been stained by the Feulgen method and examined microscopically.

In such preparations the stained nuclei of the leucocytes have a characteristic appearance. In addition, granules in freshly formed exudates and fibrous strands in cases with long standing effusion are abundantly present in the extracellular areas. Since the Feulgen staining is considered a highly specific histochemical reaction for desoxyribose nucleoprotein,[4,5] the abundance of stained material both intra- and extracellular in the micro-

[3] Dische, Z., *Mikrochemie*, 1930, **8**, 4.

‡ Dr. Milton Levy, Department of Biochemistry, New York University College of Medicine, kindly supplied the Na thymonucleate available for this experiment.

[4] Mirsky, A. E., *Advances in Enzymology*, 1943, **3**, 1.

[5] Stowell, R. E., *Stain Technology*, 1946, **21**, 137.

scopic preparations lends support to evidence obtained by chemical means that desoxyribose nucleoprotein constitutes a considerable proportion of purulent sediment that has been generally accepted as being fibrin or denatured serum proteins. The accompanying photographs illustrate the microscopic appearance of exudates of a freshly formed inflammatory effusion (Fig. 2), and of a tuberculous empyema of long standing (Fig. 3).

Up to the present time, no determinations of the presence or significance of ribose nucleoprotein have been made.

Summary. Desoxyribose nucleoprotein has been identified as a significant constituent of purulent and inflammatory exudates derived from patients. Its chemical characteristics have been described and it has been isolated in quantities ranging from 30 to 70% of the total purulent sediment.

Its physical characteristics of stringiness, viscidity, and coagulated gel, indicate its significance in contributing to the sedimented elements of exudations. Protein-free desoxyribonucleic acid has been derived from the nucleoprotein.

The liquefaction of both the nucleoprotein and the nucleic acid has been found to occur following the addition of beef desoxyribonuclease and preparations of hemolytic streptococcal filtrates containing desoxyribonuclease.

By the Feulgen method of staining the abundance of desoxyribose nucleoprotein in exudates has been demonstrated microscopically.

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Streptococcal Desoxyribonuclease: Significance in Lysis of Purulent Exudates and Production by Strains of Hemolytic Streptococci.*

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As has been indicated in the previous report,[1] purulent pleural exudates contain desoxyribose nucleoprotein as an important constituent which, together with depositions of fibrin, accounts for the gelatinous coagulum and coarse sediment that characterizes such material. In an investigation directed toward observing the action in patients of streptococcal fibrinolysin (streptokinase), the streptococcal product, substantially purified by one of us (L.R.C.) according to methods previously described,[2] was found to cause a striking and rapid change in the physical qualities of exudates both *in vitro* and also within the pleural cavities of patients suffering from various forms of purulent and fibrinous pleurisy. When it became evident that the characteristics of the specimens being altered were due to the presence of considerable amounts of nucleoprotein as well as fibrin, studies were undertaken which have revealed that hemolytic streptococci produce, as they grow, a potent desoxyribonuclease which is readily demonstrable as a secretory or excretory product in the fluid medium in which the organisms are cultivated. The effect of the streptococcal preparations has, therefore, been found to be dual in action, the purulent

* This study was supported by a grant from the National Institute of Health, United States Public Health Service.

† Supported by a grant to the Department of Microbiology, from the Life Insurance Medical Research Fund.

1 Sherry, S., Tillett, W. S., and Christensen, L. R., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 179.

2 Christensen, L. R., *J. Gen. Phys.*, 1947, **30**, 465.

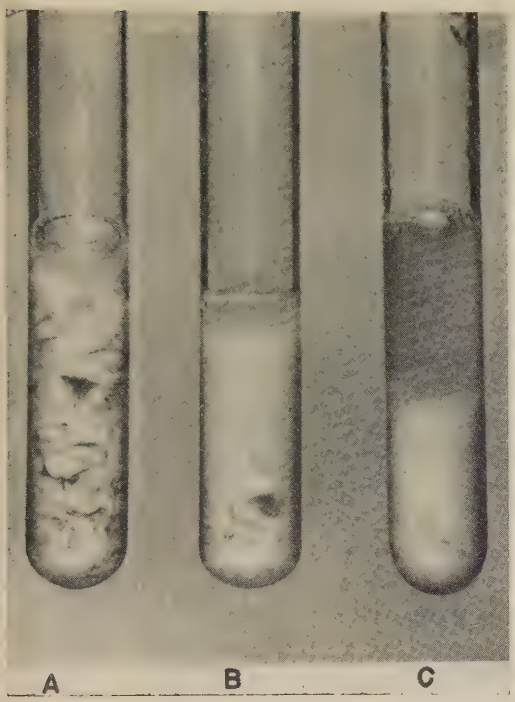


FIG. 1.
Tubes A, B, and C each contain approximately 5 cc of pus from a case of pneumococcal empyema. Tube A is an untreated control. To Tube B was added 2-3 mg of beef desoxyribonuclease. To Tube C was added 2-3 mg of crude streptococcal concentrate. The content of the tubes was mixed, shaken frequently, and observed at room temperature. Beginning changes in the character of the sediment were evident immediately. Photograph was taken after ½ hour. Tubes were uncentrifuged.

material containing two substrates, nucleoprotein and fibrin, and the streptococci yielding two substances, each acting simultaneously in a specific catalytic or enzymic manner.

Data primarily pertinent to the production of desoxyribonuclease by strains of hemolytic streptococci and the action of the enzyme on substrates of nucleic acid composition from purulent materials as well as purified preparations of animal origin‡ constitute the body of this report.

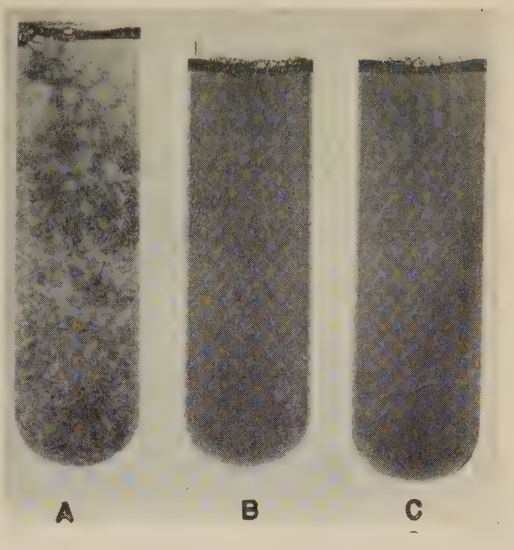


FIG. 2.
Tubes A, B, and C each contain in 7 cc the nucleoprotein isolated from 1 cc of sedimented pus by repeated extractions with 1 M NaCl. Tube A is untreated control. To Tube B was added 0.1 cc of 0.15% solution of beef desoxyribonuclease. To Tube C was added 0.1 cc of 0.15% solution of crude streptococcal concentrate. The tubes were mixed, shaken frequently, and observed at room temperature. Beginning changes in the solid strands were noted immediately and complete lysis occurred in 10 minutes. Photograph was taken after ½ hour.

Changes Induced in Pus by Desoxyribonuclease. Fig. 1 illustrates the action of: 1) beef desoxyribonuclease§ and, 2) streptococcal concentrate,¶ on the sediment of pus obtained from a case of pneumococcal empyema.

The details are given in the legend which accompanies Fig. 1.

The beef desoxyribonuclease was a well purified preparation while the streptococcal concentrate contained both desoxyribonuclease and streptokinase (streptococcal fibrinolysin). Substantial qualitative and quantitative changes in the sediment may be noted in each tube (to which the nuclease containing reagents had been added.) Following the

‡ Sodium thymonucleate (calf thymus) kindly supplied by Dr. Milton Levy, Department of Biochemistry, New York University College of Medicine.

§ The purified beef pancreas desoxyribonuclease (preparation R1MR) kindly supplied by Dr. Maclyn McCarty of the Hospital of the Rockefeller Institute for Medical Research.

¶ Lederle Laboratories have kindly prepared and supplied the concentrated filtrate from large volume broth cultures of hemolytic streptococci (Strain H46A).

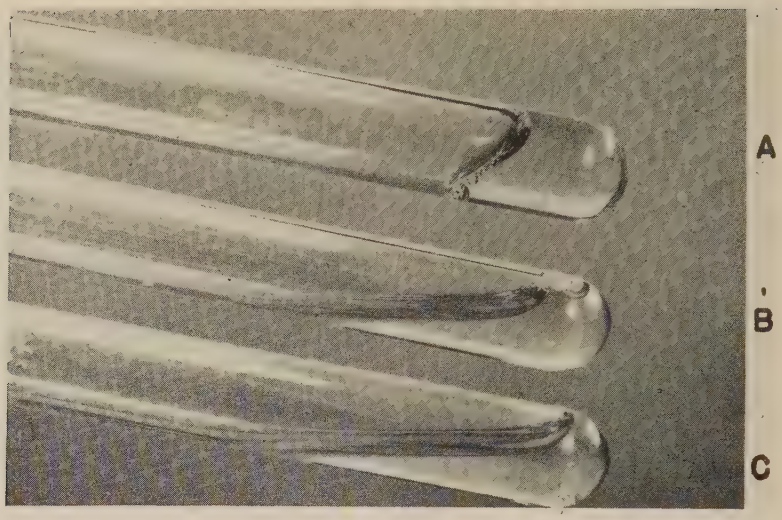


FIG. 3.

Tubes A, B, and C each contain 2 cc of 4% solution of purified desoxyribose nucleic acid (calf thymus). Tube A is untreated control. To Tube B was added 0.1 cc of 0.15% solution of beef desoxyribonuclease. To Tube C was added 0.1 cc of 0.15% solution of crude streptococcal concentrate. The tubes were mixed, shaken frequently, and observed at room temperature. Beginning changes in the gel were noted immediately. Photograph, taken with tubes on slant to indicate gelatinous quality of control substrate and complete liquefaction in other tubes, was made after ½ hour.

addition of the enzymatic substances, in each instance there was immediate thinning of the specimen, a rapid breaking up of the mucoid coagulum, a clouding of the supernatant fluid, and an obvious decrease in sediment.

Since the preparation of streptococcal origin contained at least two enzymatic elements the reduction in the sediment in Tube C is greater than that exhibited in Tube B, fibrin being liquefied in the latter as well as the nucleoprotein.

Changes Induced in Desoxyribose Nucleoprotein Derived from Purulent Exudation of a Patient with Empyema. Fig. 2 illustrates the action of: 1) beef desoxyribonuclease and, 2) a streptococcal concentrate on a sample of desoxyribose nucleoprotein isolated from human pus. The details are given in the legend which accompanies Fig. 2.

Since no fibrin was present in this specimen, the lysis of the nucleoprotein was complete on the addition of the beef desoxyribonuclease as well as the streptococcal preparation.

Alterations in the appearance of the solid strands was noted to begin immediately after the addition of the enzyme-containing preparations and dissolution was complete in a few minutes time.

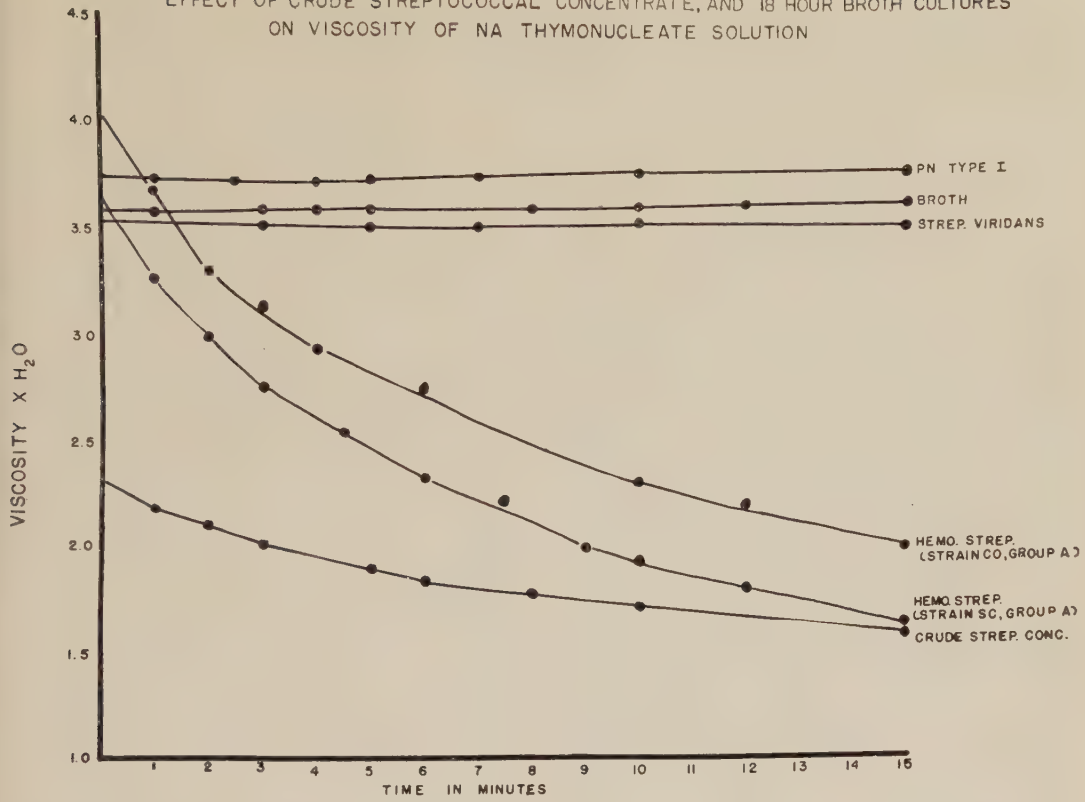
Changes Induced in Desoxyribose Nucleic Acid (Calf Thymus). Fig. 3 illustrates the action of: 1) beef desoxyribonuclease and, 2) a streptococcal concentrate on purified desoxyribose nucleic acid.

The similarity of the liquefying effect in each instance is self-explanatory. With respect to the physical character of the specimen, the gel-like quality of the desoxyribose nucleic acid may be noted while the fibrous stringy quality of the desoxyribose nucleoprotein is evident in Fig. 2.

Effect of Crude Streptococcal Concentrate on Viscosity of 1% Solution of Na Thymonucleate. From the above experiments it was apparent that the preparation of crude streptococcal concentrate contained a highly active desoxyribonuclease. In Chart 1 is shown the depolymerase activity of 0.25 cc of a 0.15% solution of the streptococcal preparation, dissolved in saline phosphate buffer, on 5 cc of a 1% desoxyribosenucleic acid solution in

CHART I

EFFECT OF CRUDE STREPTOCOCCAL CONCENTRATE, AND 18 HOUR BROTH CULTURES ON VISCOSITY OF NA THYMONUCLEATE SOLUTION



0.02 M saline containing 0.0016 M PO_4 at pH 7.20 at 37°C.

Tests For the Production of Desoxyribonuclease by Hemolytic Streptococci, Green Streptococci, and Pneumococci. The finding of desoxyribonuclease in large amounts in preparations made from filtrates of cultures of hemolytic streptococci (Strain H46A, Group C), which had been concentrated for the purpose of isolating the fibrinolytic principle (streptokinase) elaborated by the organisms, led to a study of other strains in order to determine their capacity to produce this additional enzyme. Initial interest primarily centered around the production of desoxyribonuclease in relation to streptokinase.

For this purpose several selected strains of hemolytic streptococcus were available in our laboratory in a lyophilized form which were formerly studied for the fibrinolytic activity.[3] Their source, biochemical and cultural characteristics are given in detail in Reference 3. A simple method of testing cultures directly was as follows: 5 cc of a 1% solution of a highly purified desoxyribose nucleic acid (calf thymus) in M/40 barbital buffer (pH 7.4) was placed in an Ostwald viscosimeter. Preliminary readings of viscosity were made in a water bath (37-37.5°C) for approximately 15 minutes until constant rates of flow were obtained. Then 1 cc of an 18-hour broth culture of the test strain was added and mixed. Subsequent viscosimetric readings were then made at 2, 5, 10, 15 minutes. Examples of nuclease and non-nuclease producing strains are given in Chart I.

A total of 22 strains have been tested in the manner described. When the result listed in the tabulation below is described as positive the rate and degree of change in viscosity was comparable to that given for the 2 positive strains graphically recorded in Chart I.

[3] Tillett, W. S., *J. Bact.*, 1935, **29**, 111.

Strain	Streptokinase	Nuclease
Streptococcus hemolyticus (Group A), Co, Se, Ra, Mac, OT, C108, E22, V10. (8 strains)	All positive	All positive
Streptococcus viridans from cases of bacterial endocarditis. (6 strains)	All negative	All negative
Streptococcus hemolyticus (Group B), K158A (1 strain)	Negative	Positive
Streptococcus hemolyticus (Group C), H46A, K104. (2 strains)	H46A positive K104 negative	H46A positive K104 negative
Streptococcus hemolyticus (Group E), K128. (1 strain)	Negative	Negative
Pneumococcus (1 strain each, Types I, II, VIII, XIX)	"	"

The strains tested are shown above.

From the above data it may be seen that all of Group A, fibrinolytic strains which are classified as human pathogens were also highly active in the production of desoxyribonuclease. In scattered tests with other strains of hemolytic streptococci of other serological groups, one strain of Group B proved to be active in nuclease production but without fibrinolytic activity. The strains of green streptococci and pneumococci were negative under the conditions of the method employed. In studies of the type transforming principle McCarty and Avery[4] have reported the presence of desoxyribonuclease in autolysates of pneumococcus. Under the conditions of the experimental procedure employed in this study, however, the nuclease was not found to be elaborated into the fluid medium in which the organisms were cultivated.

1. When mixtures of the two agents present in the streptococcal concentrate were heated at 56° C for one hour, the nuclease was destroyed and the streptokinase activity remained unchanged.

2. Preparations have been obtained which showed 10 fold and even greater differences in fibrinolytic titre but a constant level of depolymerase activity per unit of time.

3. Beef desoxyribonuclease was without fibrinolytic activity against either human or beef plasma clots.

4. Mg++ markedly accelerated the nuclease activity of the streptococcal concentrate but did not affect the streptokinase in a similar manner. This result is similar to the findings of others with depolymerases of animal origin.[5,6]

Summary. In experiments in which the following substrates were employed: 1) specimens of purulent exudations from patients, 2) desoxyribose nucleoprotein isolated from the exudates and, 3) desoxyribose nucleic acid derived from nucleoprotein, striking and rapid lytic changes were demonstrable in the coarse sediment of substrate 1, in the fibrous material of substrate 2, and in the gel of substrate 3, following the addition of concentrated filtrates obtained from cultures of hemolytic streptococci. The lytic action was found to be due to the presence of desoxyribonuclease which is elaborated during the growth of strains of hemolytic streptococci and is freely demonstrable in the fluid medium in which the organisms are cultivated.

Among the strains tested, hemolytic streptococci of Group A which also possessed fibrinolytic properties were uniformly potent in the production of desoxyribonuclease. By the methods of simple testing that were employed, strains of *streptococcus viridans* and pneumococcus were not found to possess a comparable property.

Desoxyribonuclease and streptokinase, produced by the same strains were found to be different substances. When streptococcal concentrates were added to whole samples of inflammatory exudates, the rapid lysis of both fibrin and desoxyribose nucleoprotein occurred.

[4] McCarty, M., and Avery, O. T., *J. Exp. Med.*, 1946, **83**, 97.

[5] McCarty, M., *J. Gen. Phys.*, 1946, **29**, 123.

[6] Carter, C. E., and Greenstein, J. P., *J. Nat. Cancer Inst.*, 1946, **7**, 29.

16430

Effect of the Intravenous Administration of Human Serum Albumin on Renal Function.*

WALTER H. CARGILL. (Introduced by E. A. Stead, Jr.)

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The intravenous infusion of concentrated human serum albumin often produces a transient diuresis in patients with nephrosis and Laennec's cirrhosis.[1,2] It has not been definitely shown that this diuresis is related to an increase in the oncotic pressure of the blood, since the immediate result of albumin administration is an increase in plasma volume and, therefore, of total circulating protein but with little or no change in the albumin or total protein concentration of the plasma.[3] The possibility exists that the administration of albumin may be followed by an alteration in renal function. We have attempted to study the immediate effect of the rapid injection of a large amount of albumin on glomerular filtration rate and renal blood flow.

Methods. The clearance techniques of Smith, Goldring and Chasis[4] have been followed, using inulin and sodium para-aminohippurate (PAH). In addition, samples of renal venous blood have been obtained by the catheterization procedure of Warren, Brannon, and Merrill,[5] and compared with simultaneous samples from the femoral artery for PAH content. The fraction of PAH extracted from the blood during passage through the kidney has been determined by the formula: $Ext_{pah} = A\text{-}RV/A$, where A and RV represent the concentration of PAH (mg %) in arterial and renal venous blood. Renal plasma flow (RPF) has been determined from the clearance and extraction of PAH ($RPF = C_{pah}/Ext_{pah}$) and this value adjusted by the hematocrit reading to obtain the renal blood flow. Glomerular filtration rate was estimated from the inulin clearance (C_{in}), and the filtration fraction (FF) from the ratio C_{in}/RPF. It will be noted that this derivation of the filtration fraction differs from that usually reported, where it is calculated from the inulin and PAH clearances alone, and since the actual renal plasma flow is always greater than the PAH clearance, our values for FF are, therefore, lower than those commonly accepted.

Following two control clearance periods 300 cc of a 25% solution of salt-poor human serum albumin† were injected intravenously in from 10 to 25 minutes. The bladder was then emptied, the urine discarded, and the experiment continued for three additional periods. Three normal subjects, three patients with hypertension, one patient with chronic glomerulonephritis without edema, and one patient with the nephrotic syndrome of subacute glomerulonephritis have been studied in this manner. In addition, similar clearance studies without catheterization of the renal vein have been performed on 2 normal subjects and one patient with the nephrotic syndrome.

Results. An immediate increase in plasma volume after the injection of albumin was indicated by a fall in the hematocrit reading of all subjects. In all but one of the subjects, (J.M.) there was an immediate increase in both the inulin and PAH clearances. In the 8 subjects from whom samples of renal venous blood were obtained, a decreased PAH ex-

* This work was supported by a grant from the Life Insurance Medical Research Fund.

1 Thorn, G. W., Armstrong, S. H., Jr., Davenport, V. D., Woodruff, L. M., and Tyler, F. H., *J. Clin. Invest.*, 1945, **24**, 802.

2 Thorn, G. W., Armstrong, S. H., Jr., and Davenport, V. D., *J. Clin. Invest.*, 1946, **25**, 304.

3 Luetscher, J. A., Jr., *J. Clin. Invest.*, 1944, **23**, 365.

4 Smith, H. W., Goldring, W., and Chasis, H., *J. Clin. Invest.*, 1938, **17**, 263.

5 Warren, J. V., Brannon, E. S., and Merrill, A. J., *Science*, 1944, **100**, 108.

† Obtained from the American Red Cross.

traction was found after albumin administration, so that the actual renal plasma flow showed a greater rise than would be indicated from the PAH clearance alone. The fall in PAH extraction in patient J.M. more than compensated for the slight decrease in PAH clearance, so that he also showed an increased plasma flow. Since the renal plasma flow rose more than the filtration rate, there was a fall in the filtration fraction of all subjects. In spite of the marked drop in hematocrit readings, there was an increase in renal blood flow in all subjects.

The average increase in filtration rate for the 11 subjects was 13% of the initial value. The control filtration rates of the 2 patients with the nephrotic syndrome (G.W. and M.B.) were below normal, 66 and 77 cc/min., and following albumin, these rose to 110 and 129 cc/min., increases of 67 and 68%. The increases in renal plasma flow ranged from 35 to 106%, averaging 58% for the group of 8 subjects in which it was measured. A significant increase in the rate of urine flow was seen in only one patient, G.W., who had nephrosis and massive edema. All of the subjects were undergoing a water diuresis at the time of study, however, to facilitate the clearance determinations.

The individual data are tabulated in Table I. Fig. 1 illustrates the changes produced in a patient with nephrosis. Fig. 2 demonstrates the individual variations in the degree and duration of the decreased PAH extraction, and includes 6 additional subjects in whom PAH extraction was determined at intervals for similar periods of time but to whom no albumin was given. These control subjects demonstrate that water diuresis and renal vein catheterization do not in themselves cause changes in PAH extraction.

Discussion. Although the number of patients studied is too small to allow generalization, several factors of interest may be noted. It is apparent that the administration of albumin is followed by definite changes in renal hemodynamics, and any attempt to explain the diuretic action of albumin must take this into consideration.

These studies also emphasize the danger of

TABLE I.
Determinations of Renal Function Before and After the Injection of 75 Grams of Albumin.

Pt.	Diagnosis	C_{IN} Before	C_{IN} After	C_{PAH} Before	C_{PAH} After	% Ext. PAH Before	% Ext. PAH After	RBF Before	RBF After	FF Before	FF After
H.K.	Normal	123	131	622	915	—	—	—	—	—	—
B.W.	"	143	150	692	850	—	—	—	—	—	—
M.A.B.	"	165	189	863	1123	82.7	71.2	1921	2429	.15	.13
W.W.	"	112	124	741	1001	90.4	78.3	1456	2110	.14	.10
R.A.	"	130	138	638	764	81.0	61.0	1401	1995	.17	.11
M.S.	Hypertension	109	112	375	491	87.3	74.3	700	994	.25	.15
E.F.	"	100	103	542	693	82.5	73.7	1177	1450	.15	.11
B.H.	"	36	39	145	154	58.0	43.6	247	398	.21	.13
J.M.	Chronic nephritis	56	32	265	240	64.0	45.1	636	783	.14	.05
M.B.	Nephrotic syndrome	77	129	498	841	—	—	—	—	—	—
G.W.	"	66	110	444	757	90.0	75.0	816	1604	.13	.11

All figures are the averages of at least 2 clearance periods. Clearances have been corrected to 1.73 sq.m. surface area.

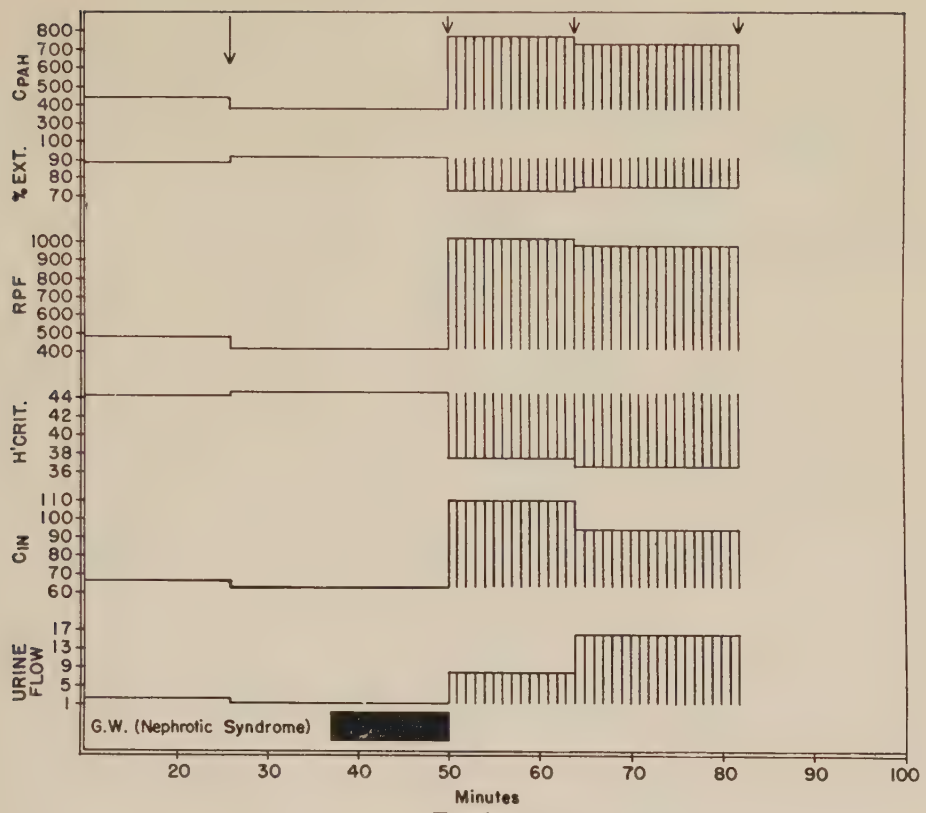

FIG. 1.

Schematic representation of the effects of albumin administration. The black bar represents the duration of injection of 75 g of albumin. The arrows indicate the end of each clearance period.

studying the effect of a substance on renal blood flow by means of the PAH clearance alone. The effect of the substance on the tubular extraction of PAH must also be determined. Since albumin actually increased the urinary excretion of PAH in the experiments reported here, a decreased extraction would have appeared unlikely. The data show that conclusions as to the extraction of PAH drawn from the rate of excretion of PAH may be in error.

There are several possible explanations for the decreased extraction of PAH. Functional impairment of the cells of the proximal convoluted tubules must be considered. It is conceivable that the injection of albumin led to its increased filtration and subsequent reabsorption, thereby over-loading the transfer mechanism of the tubular cells. A more likely explanation, however, is that accessory vascular channels were opened to accommodate the increased volume of blood perfusing the kidney, analogous to changes which have been observed in cutaneous vessels during saline infusions.[6] Morphologic studies have demonstrated the presence of juxta-medullary arterio-venous communications in the human kidney;[7] diversion of a portion of the renal blood flow through this pathway could account for the decreased extraction of PAH and the decreased filtration fraction observed. It is possibly significant that the 2 patients with advanced renal disease (J.M. and B.H., represented by the lowermost lines in Fig. 2)

[6] Altschule, M. D., Freedberg, A. S., and McManus, M. J., *Arch. Int. Med.*, 1947, **80**, 491.

[7] Trueta, J., Barclay, A. E., Daniel, P. M., Franklin, K. J., and Prichard, M. M. L., *Studies of the Renal Circulation*, Chap. IV, Charles C. Thomas, Springfield, 1947.

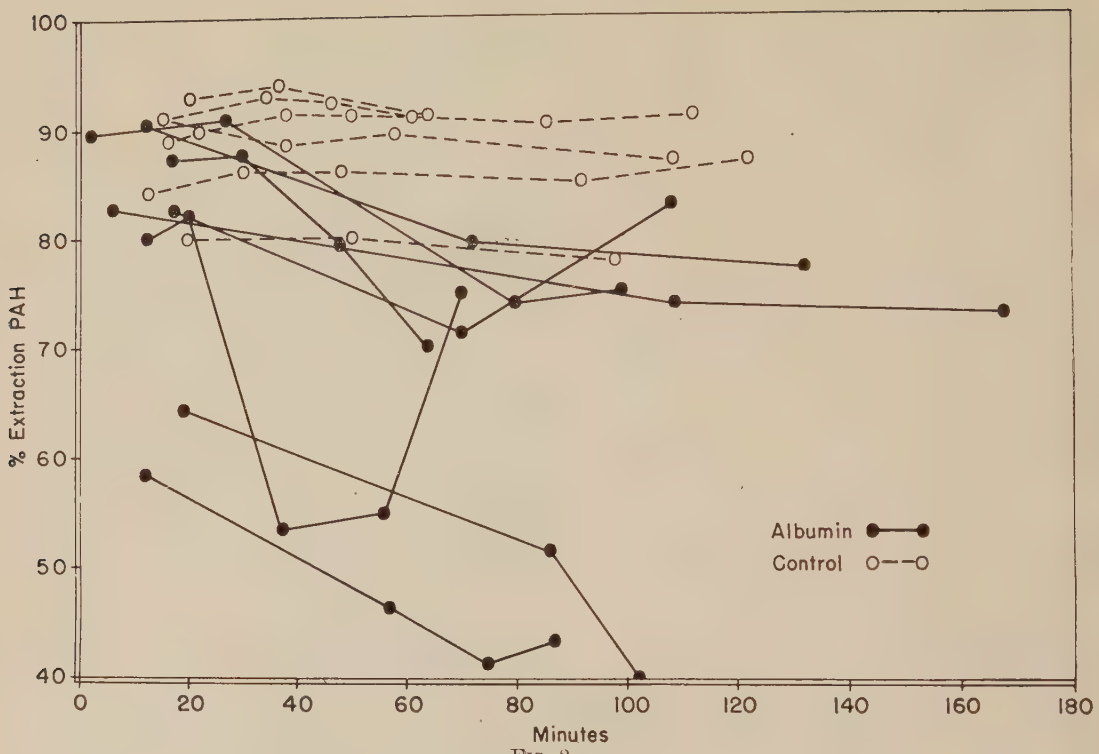

FIG. 2.

The fall in PAH extraction after albumin. Each point represents the simultaneous collection of arterial and renal venous samples.

showed the greatest and most prolonged fall in PAH extraction following albumin, since juxtamedullary shunts are most prominent in the granular, contracted kidneys of chronic nephritis and nephrosclerosis.[7]

The apparent decrease in the extraction of PAH might be due to a change in the rate of its acetylation. Determinations of total as well as free PAH after albumin have eliminated this possibility.

Summary. 1. The rapid intravenous infusion of 75 g of human serum albumin produced a consistent increase in glomerular filtration rate and renal blood flow. These changes were accompanied by both an increased clearance and a decreased tubular extraction of sodium para-aminohippurate. 2. In 2 patients with the nephrotic syndrome of chronic glomerulonephritis the administration of albumin increased the filtration rate from low to normal levels, and in one of these patients produced a marked diuresis. 3. A possible mechanism for the decreased tubular extraction of PAH accompanying an increased renal blood flow is presented.

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Experimental Attempts to Transmit Phlebotomus (Sandfly, Pappataci) and Dengue Fevers to Chimpanzees.*

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As part of a study designed to test the susceptibility of chimpanzees *(Pan satyrus)* to certain human viruses, the infectivity of phlebotomus (pappataci, sandfly) fever virus and of dengue virus was tested in this species of animals. The primary object of these experiments was to determine whether clinical or subclinical infection could be established in this species of primates and, if so, whether any recognizable symptoms or signs of these diseases could be produced.

A. Phlebotomus (Sandfly, Pappataci) Fever. History of Experimental Infections. To our knowledge this virus has never been proved to infect any laboratory animal.[1]

Strain of Virus Used. The strain of virus used was isolated in Cairo, Egypt, in 1943, from blood samples taken from a series of patients in the acute phase of sandfly fever. It has been termed the *Middle East strain.* Its properties have been previously described and it has been used extensively to produce the experimental disease in human volunteers both in this country and abroad.[1] Previous attempts had been made to infect animals with this strain by the inoculation of serum or blood of proven infectivity for human beings, by the intracerebral, intracutaneous, subcutaneous, intratesticular, intranasal or intraperitoneal routes, in the following animals; young baboons *(Papio hamadryas);* several varieties of Cercopithecus and rhesus monkeys, and various rodents, including white mice, wild gray mice, Syrian hamsters, Egyptian desert rats (jerboas), rabbits, guinea pigs and cotton rats. No evidence of pathogenicity was found in these animals. The virus could not be demonstrated in the serum of 3 rhesus monkeys 3 to 4 days after inoculation in attempts made by one of us (A.B.S.) by subinoculation in human volunteers of proved susceptibility. Material used for inoculating the chimpanzees in this experiment represented a pooled lot, totalling 7.4 ml of human serum collected from natural cases, and which had been kept frozen in 5 different ampoules (with occasional thawing in some) for about 3 years. Inoculations were given both subcutaneously (0.5-0.9 ml for each) and intracutaneously (0.5-0.9 ml for each). It should be pointed out that one of us (A.B.S.) has found human serum to be infective after 4 years of storage on dry ice.

Number of Animals. Six chimpanzees were used†—Webb, Flora, Jent, Nina, Maria and Pinta. All of these animals had been previously used for experimental work in poliomyelitis,[2] but none were considered to be "infective" as far as poliomyelitis was concerned, at the time the sandfly fever experiments were begun. Only 2 of the animals were sufficiently tractable to allow temperatures to be taken. These temperatures were taken daily when possible.

Results. The period of observation lasted

* This investigation was conducted by the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of The Surgeon General, U. S. Army, Washington, D. C.

1 Sabin, A. B., Philip, C. B., and Paul, J. R., *J.A.M.A.*, 1944, **125**, 603.

† Several of the animals used in this and the experiment on dengue were generously made available to us by the Yerkes Laboratories of Primate Biology, Orange Park, Florida. We are indebted to Dr. K. S. Lashley for his assistance in providing these animals.

2 Melnick, J. L., and Horstmann, D. M., *J. Exp. Med.*, 1947, **85**, 287.

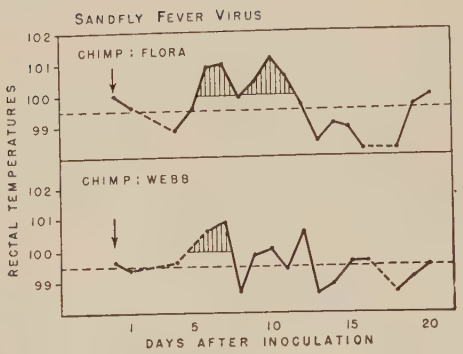

FIG. 1.

20 days. None of the 4 inoculated animals on which temperatures were not taken, showed any noticeable symptoms nor was there evident malaise or loss of appetite.

On the 2 animals in which temperatures were taken, the story is as follows:

Webb—During a 15-day-period prior to inoculation, the rectal temperature had maintained a fairly flat level ranging between 99° and 100.1°, which is normal for the chimpanzee. Following inoculation on 24 October 1946, the temperature continued normal for about 5 days to be followed by a rise of 0.5° to 1°. This slight fever was irregular with 2 humps, spaced about 4 days apart (Fig. 1).

Flora—The rectal temperature prior to inoculation had also ranged between 98.6° and 100.1°. and beginning 5 days after inoculation, there was a rise reaching 1.2° of fever, and lasting in all about 6 days. During this period, the animals seemed quiet and somewhat dull on two occasions; during the end of the febrile period the eyes had a slightly glazed appearance.

The slightly elevated temperature observed in the 2 animals appeared after an incubation period of 5 days and although irregular was protracted over a period of 6 to 7 days. We did not attempt to demonstrate viremia in these chimpanzees during their febrile period by inoculating human volunteers with chimpanzee blood. Furthermore, a test for antibodies for sandfly fever virus is not yet available and so this determination was not done. Therefore, we cannot be sure that the fever exhibited by these two animals was due to the inoculation of virus. While the course of this fever and the length of the incubation period in the 2 chimpanzees might be comparable with that of human sandfly fever,‡ it is also possible that the fever may have been due to serum disease. One of us (A.B.S.) observed fever of 2 to 3 days' duration in human volunteers who were inoculated with serum of rhesus monkeys used in tests on sandfly fever, but the usual changes in the leukocytes seen in natural and experimental sandfly fever were absent, passage to other human volunteers was negative, and the original volunteers were subsequently found to be susceptible to the virus. No conclusion is possible, therefore, concerning the susceptibility of chimpanzees to the virus of sandfly fever.

B. Dengue Fever. History of Experimental Dengue Infections. In laboratory animals experimental infections with dengue virus apparently have never been very dramatic except after adaptation in mice[3] and in certain rhesus monkeys inoculated intracerebrally with mouse-adapted virus.[4] Simmons, *et al.*[5] found that it was possible to produce inapparent infection in *Macacus philippinensis* monkeys caught at elevations above 4000 feet in the dengue-free mountains of the Philippine Islands, and in *M. fuscatus* monkeys imported from Japan. Blanc and his coworkers[6] and Findlay[7] have reported similar results in Asiatic and African monkeys. By subinoculation tests in human volunteers, one of us (A.B.S.) in association with Dr. Max Theiler (unpublished experiments) demonstrated inapparent infection in rhesus monkeys inoculated intracerebrally or intraperi-

‡ In humans the incubation period ranges in experimental cases from 2½ to 7 days. The febrile course of the disease in the great majority of cases ranges from 2 to 4 days.[1]

3 Sabin, A. B., and Schlesinger, R. W., *Science*, 1947, **101**, 640.

4 Sabin, A. B., Paper presented at meeting of Am. Soc. of Trop. Med., December 3, 1947.

5 Simmons, J. S., St. John, J. H., and Reynolds, F. H. K., *Philippine J. Sc.*, 1931, **44**, 1.

6 Blanc, G., Caminopetros, J., Dumas, J., and Saenz, A., *C.R. Acad. Sci., Paris*, 1929, **188**, 468.

7 Findlay, G. M., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1932, **26**, 157.

toneally with the strain of human dengue virus used in the present experiments. Subsequently one of us (A.B.S.) also found that rhesus monkeys inoculated with human dengue virus developed neutralizing antibodies for the mouse-adapted virus.

Strain of Virus Used. The Hawaii strain of dengue virus recovered by one of us (A.B.S.) in 1944 from natural cases of the disease in Hawaii was used in the present tests. Serum collected within less than 24 hours after onset of fever from the first group of human volunteers inoculated with the virus from the natural cases, and stored in the lyophilized state in an ordinary refrigerator since 17 April 1944 was used in the first experiment on 11 December 1946. Serum obtained within 24 hours after onset of fever in other human volunteers, inoculated with subsequent passages of this virus, and stored in a box containing solid CO_2 since 1944, was used 3 years later in the second experiment on 31 July 1947. Recent tests carried out by one of us (A.B.S.) on human volunteers showed that dengue virus in human serum stored for more than 3 years either in the frozen state in a box containing solid CO_2 or in the lyophilized state in an ordinary refrigerator was still infectious.

Number of Animals. Nine chimpanzees from 2 to 5 years of age were used in these experiments. Several of them had been used previously for experimental work in poliomyelitis,[2] Fort Bragg (pretibial) fever,[8] and sandfly (phlebotomus) fever. Five of these animals were tractable enough to have their temperatures recorded daily. However, all animals were observed daily for signs of illness and evidence of a rash for a period of 4 to 5 weeks.

Technique of Neutralization Tests. All of the neutralization tests were carried out by one of us (A.B.S.). Through them it was possible to confirm the presence of subclinical infection in the inoculated chimpanzees, by the use of the neutralization test developed by Sabin and Schlesinger following their adaptation of the Hawaii strain of dengue virus in mice.[3] The technique of the test§ and the extensive observations made by these investigators on the development of antibodies in human volunteers, natural cases and experimental animals have not as yet been published. The essentials of the test are: 1) that the sera to be tested be maintained in the frozen state; 2) that the original suspension of dengue-infected mouse brain as well as the dilutions be prepared in undiluted rabbit serum which had been heated at 56° C for 30 minutes; and 3) that the serum-virus mixtures be incubated in a water bath at 37° C for 2 hours prior to intracerebral inoculation in mice. In the present study the pre-inoculation and post-inoculation serum specimens were kept frozen in a box containing solid CO_2, and tested simultaneously.

Results. Exp. 1—Dec. 11, 1946. Four animals (Webb, Jent, Flora and Pinta) were each inoculated subcutaneously with 0.5 ml of human dengue serum. This was from a lyophilized sample which had been collected on 17 April 1944. It was redissolved on the day of the chimpanzee inoculations. Daily temperatures are recorded for 2 of the animals, Webb and Flora, and one of them, Flora, had a slight fever on the 9th day (Fig. 2). There were no other signs of disease.

Neutralization tests on matched specimens of preinoculation and postinoculation serum were carried out in one animal, Webb.

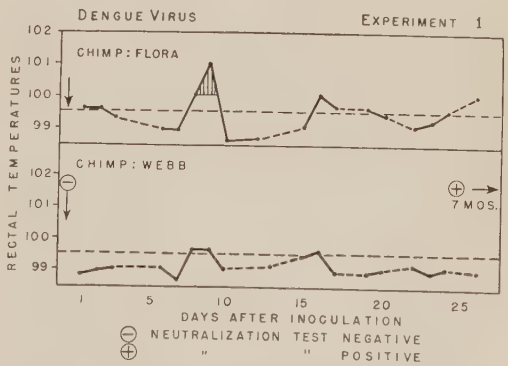

FIG. 2.

[8] Melnick, J. L., and Paul, J. R., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 263.

§ See Sabin, A. B., in *Procedures for Virus and Rickettsial Diseases*, 1948, p. 289, Am Pub. Health Assn., New York City.

TABLE I.

Development of Neutralizing Antibodies in Chimpanzees Inoculated with Human Dengue Virus (Hawaii Strain)

Virus Used—59th mouse brain passage of Hawaii Dengue.

Serum tested	Mortality at indicated dilutions of virus					LD_{50} Log of Dilution	Neutralization Index*
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
Control—undiluted rabbit	—	5/5	3/5	3/5	1/5	5.0	—
Webb (11-2-46)—before dengue	5/5	5/5	5/5	2/5	—	4.8+?	2—?
" (7-19-47)—7 mos. post dengue	0/5	0/5	0/5	0/5	—	1.5—?	3,200+?
Rosebud (7-31-37)—before dengue	5/5	5/5	5/5	4/5	—	5.4+?	—3
" (9-23-47)—54 days post dengue	0/4	1/5	0/5	0/5	—	1.6—?	2,500+?
Hickory (7-31-47)—before dengue	5/5	5/5	4/4	1/5	—	4.6+?	3—?
" (9-23-47)—54 days post dengue	1/5	0/5	0/5	0/5	—	1.6—?	2,500+?
Becky (7-31-47)—before dengue	5/5	5/5	5/5	2/5	—	4.8+?	2—?
" (9-23-47)—54 days post dengue	3/5	0/4	0/5	0/5	—	2.2—?	630+?
Catawba (7-31-47)—before dengue	5/5	5/5	5/5	5/5	—	5.5+?	—3
" (9-23-47)—54 days post dengue	1/5	0/2	0/5	0/5	—	1.6—?	2,500+?
Mary Lou (7-31-47)—before dengue	5/5	5/5	5/5	5/5	—	5.5+?	—3
" " (9-23-47)—54 days post dengue	0/2	1/4	0/2	0/4	—	1.7—?	2,000+?

* The neutralization indexes were calculated on the basis of the control titer ($10^{-5.0}$) obtained with heated undiluted rabbit serum.

Neutralizing antibodies, absent before inoculation, were present in the convalescent specimen. See Table I.

Experiment 2—July 31, 1947. Tests with ultracentrifuged fractions. 100 ml of human serum, frozen since 1944, were thawed and spun at 15,000 r.p.m. for 30 minutes in the cold in the RR-1 International centrifuge. The fatty cake was discarded and the aqueous phase spun in 12 tubes in the ultracentrifuge (10° angle rotor) at 33,000 r.p.m. for 90 minutes with a force of 60,000 x gravity at the middle of the tubes.

No visible sediment was obtained. The top 7.2 ml of each tube was drawn off and pooled. The bottom 0.6 ml of each tube was then rubbed against the sides and bottom of the tube with a rubber tipped glass rod. The bottom fractions were then pooled. Nitrogen determinations (micro-Kjeldahl) were carried out in duplicate as an indication of the amount of serum proteins sedimented under these conditions, and these were as follows:

	N mg/ml
Serum before ultracentrifugation	12.3
Top fraction (86.4 ml)	11.6
Bottom fraction (7.2 ml)	17.8

The top fraction was inoculated subcutaneously (1 ml) and intracutaneously (1 ml) into each of 2 chimpanzees (Catawba and Mary Lou) and the bottom fraction by the same routes and in the same volumes into each of 3 (Rosebud, Becky and Hickory). Daily temperatures were taken on the latter 3 animals over a period of 5 weeks. (Fig. 3). No clinical signs of illness were manifest in any of the 5 animals other than a slight fever, but in examining the temperature charts carefully (Fig. 1 and 2) one can perhaps see that in all inoculated animals there is a consistent, postinoculation trend with a slight elevation of temperature at some time between the 5th and 10th days after inoculation, to be followed by a secondary slight elevation from the 12th to the 17th days. This temperature pattern does not resemble the diphasic temperature occasionally seen in human dengue.

Neutralization tests were carried out simultaneously on the chimpanzee sera collected on 31 July, prior to the virus inoculations, and 54 days later on 23 September. All 5 animals responded to the inoculation with the development of neutralizing antibodies in high titre (Table I).

Discussion. Six chimpanzees inoculated with human serum, believed to contain phlebotomus fever virus, exhibited no signs of illness, other than fever which was measured in but 2 of them and the very slight temperature elevation which occurred in the 2 animals whose temperature was recorded, cannot be interpreted with certainty. In the absence of

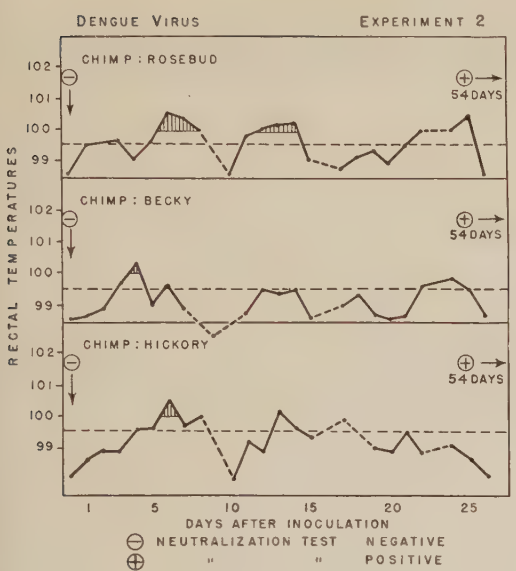

FIG. 3.

leucocyte studies and subinoculation tests in human volunteers, there is no evidence to indicate one way or another, whether chimpanzees are susceptible to this virus.

Among 9 chimpanzees inoculated with human dengue virus, there was again no clinical evidence of infection or striking elevation in temperature. However, by means of the neutralization test developed by Sabin and Schlesinger it was found that all those tested developed neutralizing antibodies for the homologous mouse-adapted dengue virus, and this may be interpreted as indicating that an inapparent infection followed the inoculation of human dengue virus in chimpanzees. Sabin and Schlesinger (unpublished experiments) found that in unsusceptible animals such as hamsters, cotton rats, guinea pigs and rabbits, a single inoculation of a large dose of dengue virus is not followed by the development of neutralizing antibodies, while inapparent infections resulting from single inoculations of mouse-adapted virus in rhesus monkeys or human beings invariably give rise to these antibodies. It is of interest that none of the 6 chimpanzees tested had any antibody for the Hawaii strain of dengue virus used in these tests prior to inoculation and that all developed antibodies of high titre after inoculation. The persistence of the neutralizing antibody in high titre for at least 7 months is in keeping with observations one of us (A.B.S.) has made on the long persistence of dengue antibody in human beings.

Previous experiments, not yet reported in detail, carried out by one of us (A.B.S.) in association with Drs. R. W. Schlesinger and Wendell M. Stanley, on the ultracentrifugation of human dengue virus,[3] indicated that the virus can be concentrated in the sediment obtained by centrifugation at 24,000 r.p.m. (50,000 x gravity at middle of tubes) for 90 minutes in a 33° angle, 8-inch rotor. In the present tests it is of interest that centrifugation at 33,000 r.p.m. (60,000 x gravity) for 90 minutes in a 10° angle 6½-inch rotor did not yield any visible sediment although under these conditions an appreciable fraction of the serum proteins sedimented into the bottom part of the tubes. It should be recalled that before being subjected to ultracentrifugation, these sera had been frozen and thawed, and then spun at 15,000 r.p.m. for 30 minutes. The fact that after ultracentrifugation the top fraction produced neutralizing antibodies in the 2 chimpanzees inoculated with it, as well as did the bottom fraction in three other chimpanzees, need only mean that a certain amount of virus remained unsedimented. Other viruses centrifuged in such angle rotors have also been found to remain in small amounts in the supernatant liquids.[9,10]

Summary. 1. Six chimpanzees inoculated with human serum believed to contain phlebotomus (sandfly, pappataci) fever virus, exhibited no clinical signs of infection. A slight febrile response in the 2 chimpanzees, whose temperatures were being recorded, could not be interpreted with certainty, and no evidence, pro or con, was adduced regarding the susceptibility of chimpanzees to this virus.

2. Nine chimpanzees inoculated with human dengue virus (Hawaii strain) also exhibited no clinical signs of infection, but

[9] Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1936, **64**, 503.

[10] Melnick, J. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 553.

evidence of inapparent infection was obtained by neutralization tests with the homologous mouse-adapted virus. None of 6 chimpanzees, whose serum was tested, had any antibodies for the dengue virus before inoculation and all developed them in high titre after inoculation.

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Accidental Laboratory Infection with Human Dengue Virus.*

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Accidental laboratory infections with a wide variety of different viruses including viscerotropic yellow fever virus and Rift Valley fever[1] virus have frequently occurred. No such infection with dengue virus, however has previously been recorded. It is the purpose of this paper to present observations upon a patient with dengue who acquired this disease by accident in the laboratory.

The patient, a 23-year-old male negro laboratory assistant was exposed to infection under the following circumstances. In July, 1947 he participated in one of the experiments (Experiment No. 2) which were described in the previous paper on the transmission of dengue to chimpanzees.[2] It will be recalled that a pooled sample of infected human serum obtained in 1944 in Hawaii from patients acutely ill with dengue and preserved in the frozen state was separated into two fractions designated "top" and "bottom" following ultracentrifugation at 33,000 R.P.M. for 90 minutes. On 31 July each fraction was injected into chimpanzees and both fractions were subsequently found to contain dengue virus. During the efforts to restrain and inoculate the chimpanzees a syringe containing the "bottom" fraction suddenly separated from the needle, and some of the serum which squirted out struck the patient in the eye. His eye was immediately washed out with water, and he resumed his work. The subsequent developments to be described are charted in Fig. 1.

The patient remained well during the next week. On 7 August he noted a macular rash distributed over the bearded area of his face. On 9 August after a good breakfast he became tired and weak on his way to work. During the morning he developed aches in the calf muscles and behind his knees as well as throbbing frontal headache. He returned home at noon, ate an egg and vomited promptly. His temperature at noon was 101°F, at 3:30 P.M., 103.6°, at 8:00 P.M., 102.6°. During the afternoon he had repeated chills. On 10 August he complained of headache, anorexia, nausea and vomiting. His eyes burned and the muscles of his legs ached. He had no symptoms referable to the respiratory tract. His wife and two children were well and had not recently been ill. On physical examination he appeared sluggish but not particularly ill. His temperature was 102.2°. The pulse and respiratory rates were not accelerated. A macular rash was present on the face as well as a few indistinct macules on the right shoulder and back. Tenderness over the eyeballs and slight injection of the conjunctivae and pharynx were noted. Small cervical and axillary lymph nodes were palp-

*This observation occurred in the course of investigations conducted by the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of The Surgeon General, U. S. Army, Washington, D. C.

1 Sabin, A. B., and Blumberg, R. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 385.

2 Paul, J. R., Melnick, J. L., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 193.

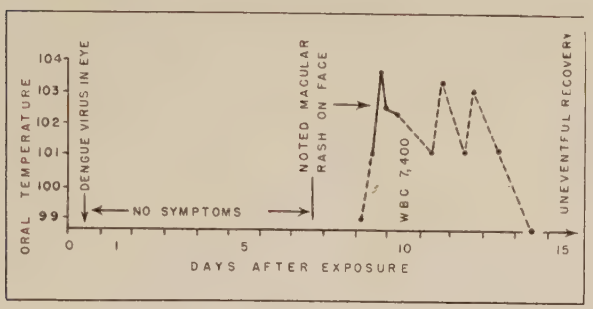

FIG. 1.

able and somewhat tender. The lungs were clear and the cardiac findings normal. The total leucocyte count was 7,400. The possibility of infection with dengue virus was considered, and blood was drawn for serological studies.

The patient remained at home and continued to have temperature elevations ranging between 101 and 103°F with aches in his head, eyes and legs. He was seen again on 13 August. The lymph nodes in the neck, axillae and both inguinal regions were larger and more tender than on the previous examination. The spleen was still not palpable and the rash had disappeared. On 14 August he felt much better and his temperature had returned to normal. The enlarged lymph nodes previously noted were still somewhat tender. His subsequent course was uneventful.

Specimens of serum were obtained at intervals 1, 13, 25, and 45 days after the onset of illness on 9 August. Neutralization tests in mice were carried out with these sera by one of us (A.B.S.) according to the technique developed by Sabin and Schlesinger which was cited in the preceding paper.[2] The dengue virus used in the neutralization tests was the 59th mouse brain passage of the Hawaii strain. The results of these tests are shown in Table I.

The titer (50% mortality end point) of dengue virus combined with normal rabbit control serum was $10^{-5.7}$. The serum from the patient obtained one day after the onset of illness contained no neutralizing antibody against this virus as indicated by the fact that the titer observed in mice inoculated with the mixture of virus and this serum was similar to that of the control. The specimens of serum obtained during convalescence, however, all appeared to contain antibody as they neutralized from approximately 8,000 to 25,000 LD_{50} of virus.

Comment. The available evidence strongly suggests that the laboratory worker had dengue as a result of the accidental introduction of virus into his eye. The fact that the eye was probably the portal of entry in this case parallels the finding that dengue can be produced in human volunteers by instilling large amounts of human virus into the con-

TABLE I.
Results of Neutralization Tests with Dengue Virus (Hawaii Strain).

Source	Serum: Days between onset and bleeding	Fate of mice following intracerebral injection of serum-virus mixture*: Dilution of virus 10-1	10-2	10-3	10-4	10-5	10-6	Titer of Virus† (LD_{50})	Neutralization Index
Normal rabbit	—	—	—	5/5	5/5	3/4	2/5	5.7	—
Patient	1	—	5/5	5/5	5/5	4/5	—	5.4+?	2—?
”	13	—	2/5	0/5	0/5	0/5	—	1.8—?	8,000+?
”	25	5/5	1/5	0/5	0/5	—	—	1.6	13,000
”	45	3/5	1/5	0/5	0/5	—	—	1.3	25,000

* Indicated by a fraction in which the denominator indicates the number of mice injected with each mixture and the numerator the number which died.
† Log of dilution.

junctival sac.[3] It seems quite clear from the experiments with chimpanzees that the serum which splashed into the patient's eye actually contained dengue virus. As New Haven is not in an area in which dengue is endemic there appears to have been no other likely source of infection and none of the patient's family or associates were concurrently ill. The interval of 9 days between the accident and the development of symptoms is not too long for the incubation period of dengue and the clinical course of the illness was compatible with that of the naturally occurring disease. The serological finding that neutralizing antibodies against the mouse-adapted Hawaiian strain of dengue virus developed in the patient's serum in high titer within two weeks after the onset of illness is, of course, strong evidence in support of the diagnosis. The fact that the patient developed an acute febrile illness under the circumstances which have been presented may be regarded as additional evidence that the virus which produced inapparent infection in chimpanzees[2] as described in the preceding paper had retained its pathogenicity for man.

Summary. In the course of experiments with chimpanzees, human dengue virus was accidentally introduced on to the conjunctival surface of the eye of a laboratory worker. This was followed 9 days later by a febrile illness, clinically compatible with dengue. Neutralizing antibodies for the homologous mouse-adapted strain of dengue virus were absent in the patient's serum taken one day after onset of fever, but were present in high titer in specimens of serum obtained 13, 25, and 45 days after onset.

3 Sabin, A. B., Unpublished studies.

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Serum Treatment of Western Equine Encephalitis in Mice Determined by the Course of Viral Infection.

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Certain features of serotherapy of experimental infections with neurotropic viruses are still under deliberation. A group of investigators maintain that animals prostrated by the infection can still be completely revived and restored to a normal-seeming state while other workers hold that once the virus multiplies actively or the host exhibits a definite syndrome reflecting the involvement of the central nervous system, *i.e.*, a destruction of tissue or neurons, then serotherapy is often ineffective. There is general agreement, however, on the successful prevention or treatment of the experimental disease if potent antiserum in sufficient amount is given early enough in its course, or, generally, before definite physical signs of infection of the central nervous system are manifest.

The problem was to determine at what stage during the course of an experimental infection antiserum was or was not effective, especially in relation to the a) time after exposure to the virus, b) first appearance of physical signs, and c) degree of multiplication of the virus in the brain. By means of the intracerebral route, 100% rate of lethal infection could be obtained with a sufficient dose of virus; and by the use of the Kelser strain of Western equine encephalitis virus which is only moderately active or invasive, the incubation period could be conveniently prolonged for serum treatment at different intervals after exposure to virus. Mice were

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† We are grateful for the technical assistance of Edna Blinder and Joan B. FitzGerald.

TABLE I.
Multiplication of Western Equine Virus (Kelser Strain) in Mouse Brain After Intracerebral Inoculation of About 2700 LD_{50}.

Hr after inoculation	Virus titer of brain tissue removed from inoculated mice*	Hr after inoculation	Virus titer of brain tissue removed from inoculated mice*	
2	$<2\times10^{-1.0}$	50	$2\times10^{-6.2}$	
6	$2\times10^{-1.5}$	54	$2\times10^{-7.0}$	
10	$2\times10^{-2.0}$	57	$2\times10^{-7.4}$	
14	$2\times10^{-3.5}$	62	$2\times10^{-7.6}$	
18	$2\times10^{-4.0}$	66	$2\times10^{-7.2}$	
22	$2\times10^{-4.5}$	70	$2\times10^{-7.6}$	
26	$2\times10^{-4.5}$	74	$2\times10^{-8.2}$	
30	$2\times10^{-5.6}$	76	$2\times10^{-7.6}$	†
33	$2\times10^{-5.8}$	80	$2\times10^{-7.0}$	
38	$2\times10^{-6.5}$	86	$2\times10^{-7.2}$	
42	$2\times10^{-6.5}$	90	$2\times10^{-7.0}$	‡
46	$2\times10^{-5.4}$	94	$2\times10^{-7.0}$	

* Brain removed aseptically; diluted with 10% normal rabbit serum in saline solution and injected intracerebrally in test mice. The LD_{50} is given in the table.
† Signs of central nervous system involvement in this period.
‡ Death due to virus in this period.

the animals of choice since large numbers could be studied thus avoiding statistical pitfalls and yet permitting the conservation of a supply of the rabbit hyperimmune serum used in the present investigation. The blood volume of a mouse of about 17 g weight—the average weight of the test mice—is 1 cc; since 2 cc of the serum were given each animal, it was therefore the equivalent of twice the animal's blood volume.‡ More than 2 cc of this type of serum was toxic by itself, reaching a rate of 100% of deaths within a few hours after 5 cc were given.

The rate of multiplication of the Kelser strain had been studied[1] by sacrificing mice at 4-hour intervals from 2 to 94 hours after intracerebral inoculation of 2700 LD_{50} of the virus. As seen in Table I, the multiplication of the virus proceeded at a fairly regular rate between the 10th and 54th hour after inoculation. After about 57 hours, the virus reached its maximum titer which it maintained until the time of death. On the other hand, visible signs of illness were first noted 74 hours after inoculation and deaths occurred at the 86th to 96th hour, or later.

Thus a base line was constructed of a) the rate of multiplication of the virus in the brain of inoculated mice, b) the time of recognizable physical signs of infection and c) the time of death of the animal. The plan was to treat infected mice with antiserum at different periods of infection, for example, when no signs were visible and no multiplication of the virus occurred; when no signs were noted but multiplication was active, and when signs of illness were plainly seen. In this way a correlation of the effectiveness of the antiserum with the degree of multiplication of virus and of recognizable signs of infection could perhaps be obtained

Preparation of Antiserum. At first rabbits were immunized by intraperitoneal injection of mouse-brain virus in amounts representing at least $10^{7.5}$ LD_{50}. For example, after 6 such injections a rabbit antiserum when diluted 1:100 exhibited a neutralizing index of 500, as shown by the intracerebral mouse-neutralization test. After an added course of 3 inoculations of mouse-brain virus, 1:1000 dilution of the rabbit antiserum completely neutralized 65 LD_{50} of virus, and 1:5000 and 1:10,000 dilution of serum still showed neutralizing effect, although not completely (2 of 5 mice died) against the same amount

‡ The blood volume was calculated from the formula of Dreyer, G., and Ray, W., (*Philos. Trans. Roy. Soc.*, London, 1911, Series B, **201**, 133) *viz.*, B.V. = Weight $\frac{2}{3}$

6.70

1 Schlesinger, R. S., unpublished observations made in this laboratory.

of virus. Beyond this point, further treatment failed to raise the level of neutralizing antibody. This is in good agreement with a prior conclusion that immunization prolonged over a period of months does not necessarily lead to antibody levels higher than those obtained by properly administered shorter courses.[2] However this may be, rabbit antiserum prepared by means of mouse-brain virus could not be used owing to the toxicity of such prodigious amounts as were given to test animals. *viz.*, twice the blood volume. Nontoxic rabbit serum was then prepared by employing as immunizing antigen embryonated hens' eggs infected via the yolk sac with the Kelser strain of Western equine encephalitis virus.‖ After 9 intraperitoneal injections of "egg" virus having a titer of $10^{-7.5}$ or higher, given over a period of a month, the pooled serum from 4-6 rabbits exhibited about the same degree of viral neutralization as antiserum prepared against mouse-brain virus just described. In a test, however, serum was used which in a dilution of 1:500 or higher could prevent infection of 5-25 lethal doses of virus injected intracerebrally into mice. This preliminary titration was made by inoculation of 1 cc of graded dilutions, up to 1:1000, of antiserum subcutaneously and followed 16 hours later by injection of a constant amount, $10^{-5.8}$–$10^{-6.0}$, of virus. A control titration of the virus by itself was included so that an exact computation of the number of doses used in the test could be obtained.

Tests. Table II serves to demonstrate examples of the tests in which mice were injected with varying small multiples of lethal doses of the virus of Western equine encephalitis and given antiserum either before, along with, or after inoculation of the virus. Each mouse received .03 cc of the virus suspension intracerebrally and 2 cc of antiserum were administered intraperitoneally. A series of mice were also treated with similar injections of normal rabbit serum as controls but as the table shows, no influence of the normal serum was exerted on the course of the experimental infection. It will be noted that the number of infective units of virus used in the test was less than that employed for determining the rate of multiplication of the virus in the brain of virus-inoculated mice and the time of development of physical signs and death in them, the data of which are shown in Table I. This was purposely planned so that the reactions to the virus by the serum-treated animals would not be greater than those in these control mice, and furthermore, the smaller doses of virus used in the test animals would weigh in favor of any preventive or curative action of the antiserum. For it will be seen (Table II) that the antiserum even in the large quantities given became less pronounced in effect when the virus was increased from 5 to 25 LD_{50}, an outcome that was not unexpected. The increase of inoculated virus from 25 to 63 doses, however, exerted little if any influence on the action of the antiserum.

There was, in addition, variation in the effect of antiserum within the same series. This variation is found now and again in immunity tests of this sort and is perhaps an indicator of the varying but unpredictable individual factors of resistance that may be brought out by the action of hyperimmune serum, such as the rate of aging, physiological or constitutional elements, or other still unknown influences which would help certain animals.

It will be observed that the antiserum became less effective as the time after exposure to the virus lengthened. Thus, up to 16 hours after the virus was given, encephalitis could generally be prevented in treated mice. From 24 to 48 hours after the inoculation of virus, some but not all the treated animals remained apparently unaffected. At 72 hours, at a time when the mice were still free from signs of illness, only an occasional test animal receiv-

[2] Olitsky, P. K., Schlesinger, R. W., and Morgan, I. M., *J. Exp. Med.*, 1943, **77**, 359.

‖ In preparing the immunizing antigen in large amounts, contamination could be reduced or eliminated by placing harvested embryos in a saline solution containing 1000 units penicillin per cc. The embryos were kept in the solution until the required number was collected and were then transferred to a Waring Blendor.

TABLE II
Treatment with Hyperimmune and Normal Rabbit Serum of Mice Infected by Intracerebral Route with Varying Amounts of Virus.

	Hyperimmune rabbit serum				Normal rabbit serum†
Virus titer	$10^{-6.7}$	$10^{-7.2}$	$10^{-7.7}$	$10^{-7.6}$	
Dilution to infect	$10^{-6.0}$	$10^{-5.8}$	$10^{-6.0}$	$10^{-5.8}$	
LD_{50} used	5*	25	50	63	
Time when antiserum was given in relation to virus	Result Dead/No. used				Result Dead/No. used
2 hr before	—	0/6	—	—	5/5
1 " "	1/7	—	—	—	—
With virus	2/6	0/6	—	1/5	9/9
4 hr after	0/7	1/6	0/5	—	4/4
8 " "	—	2/6	—	—	5/5
16 " "	0/6	2/6	2/5	—	9/9
24 " "	3/7	5/6	—	4/6	9/9
30 " "	2/7	6/6	4/6	3/5	9/9
48 " "	4/6	6/6	3/6	6/6	9/9
72 " "	5/7	6/6	6/6	—	5/5

* Antilog of difference between lines 1 and 2 to nearest whole number.

† 2 cc of normal rabbit serum was given intraperitoneally to each of a series of animals receiving intracerebrally 25 and 63 LD_{50} of virus and the results of both tests are combined here.

TABLE III.
Serum Treatment of Mice at the Time of First Definite Signs of Illness.*

Mouse No.	LD_{50} of virus injected	First sign shown after virus inoculation	Signs shown after virus inoculated in hours
1	*ca.* 6	Leg weakness	73
2	"	Tail spastic	73
3	"	Ruffled fur; slow	90
4	"	" " tremors	90
5	"	" " slow	90
6	"	Circling	93
7	"	Biting (itch)	93
8	"	Ruffled fur; slow	93
9	"	" " "	93
10	"	" " "	96
11	5	" " "	90
12	5	" " "	93

* All animals died after administration of 2 cc of antiserum given iper. at the time of the appearance of physical signs.

ing the minimal amount of virus survived. When the same amount of antiserum was given to animals when definite physical signs of illness occurred, at a time corresponding to 73 or more hours after exposure to minimal amounts, *i.e.*, about 5 or 6 LD_{50} virus, the treatment proved valueless since all treated mice died (Table III).

Discussion. There is no expediency for the acceptance of the pattern laid here for serum treatment of mice as a model for that of human beings. The infection was induced in mice by intracerebral inoculation of the virus of Western equine encephalitis which is not the way of infection in nature. The effect of serotherapy in the human disease is at present not definitely known since no controlled series of observations are as yet available; Hammon[3] states that convalescent serum therapy is probably ineffective.

In the present study mice and the intracerebral route of infection have been employed for the purpose of providing sufficiently large numbers of conveniently handled animals for several tests, and secondly, to

[3] Hammon, W. M., *Clinics*, 1945, **4**, 485.

have a more solid foundation on which to discern real and not apparent results since the intracerebral route could be counted upon to induce lethal encephalitis in every instance among controls. It therefore follows that what was concluded from the experiments with mice and the intracerebral route of inoculation might not correspond with the results of tests on larger laboratory animals, such as guinea pigs and monkeys, and on the use of peripheral (nonneural) route of inoculation of virus. Then, of necessity, smaller numbers of animals have to be used and the peripheral route of viral exposure is not always disease-producing in the control animals.

What is significant is that in the present investigation a certain dosage of antiserum which could prevent infection at one particular stage during the course of infection, when the virus was even actively multiplying, was ineffective in another, when virus was increasing, or remained stationary at a higher level, Either the antiserum was incapable of neutralizing the virus at its place of multiplication[4] or an amount of serum might have been needed that in terms of blood volume of the host was impractical to use, or changes in the tissues progressed to a degree which could no longer be influenced by serotherapy. It should be stressed, however, that the stage during which antiserum was ineffective was one in which the treated animals could still appear to be normal to the observer.

Summary. If 2 cc of rabbit hyperimmune antiserum of high titer were given intraperitoneally to mice of about 17 g weight—a prodigious amount since it represents twice the total blood volume of the host—encephalitis was prevented from developing in animals receiving intracerebrally ordinarily lethal doses of the virus of Western equine encephalitis. The preventive effect was noted when the virus was multiplying but had not as yet reached its maximal titer. Thus, if animals were treated with antiserum 2 hours before, at the same time, or from 4-16 hours after the virus was inoculated, it was effective in preventing an attack of encephalitis. Even if serum was administered 24, 36 or 48 hours after the virus certain but not all mice survived without recognizable signs of the disease. At 72 hours, however, only an exceptional mouse survived after serum treatment. Finally, at 73 or more hours after virus inoculation, when definite manifestations of experimental Western equine encephalitis could be observed, injection of antiserum was found to have no influence on the course of the lethal infection.

[4] Rivers, T. M., *J. Am. Med. Assn.*, 1948, **136**, 291.

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Penicillin Aerosol in the Treatment of Experimental Pneumococcus Pneumonia.*

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Inhalational therapy with penicillin mist, or aerosol, in acute and chronic respiratory infections in man has recently been receiving the attention of clinical investigators both here and abroad.[1-10] In fact, the clinical

* Work done under a grant from the Medical Division of the Chemical Warfare Service, Edgewood Arsenal, Md.

[1] Garthwaite, B., and Barach, A. L., *Am. J. Med.*, 1947, **3**, 261.

[2] Olsen, A. M., *Proc. Mayo Clinic*, 1945, **20**, 184.

[3] Segal, M. S., and Ryder, C. M., *New Eng. J. Med.*, 1945, **233**, 747.

application of this method of administration of the drug has outdistanced controlled animal experiments designed to prove its effectiveness as a prophylactic or therapeutic measure. Barach and co-workers[10] were the first to show, in experiments on rats infected intraperitoneally with a fatal dose of hemolytic streptococci, that inhalations of penicillin aerosol considerably reduced the percentage of mortality as compared with that of an untreated group. The degree of protection was not so great, however, as that conferred by intramuscular injection of the drug. Wilson and collaborators[11] in studies on experimental pneumonia which were concurrent with ours, reported that inhalations of penicillin aerosol at 6, 12, and 24 hours after intrabronchial infection of rats with pneumococci caused survival rates which compared favorably with those resulting from intramuscular injection of similar doses of the drug (97% and 100% respectively). We briefly reported[12] the results of experiments in which the prophylactic and therapeutic effect of penicillin aerosol on pneumococcus pneumonia in rats was determined. The present report includes a description of methods used by us for the production of pneumonia in rats and for the administration of the aerosol, as well as an elaboration of the results of treatment at varying times both before and after infection.

Methods. Method of Producing Lobar Pneumonia in Rats. The method of Nungester and Jourdonais[13] for intrabronchial inoculation of mucin-suspended pneumococci was used, with some modifications which in our experience simplified the technique. The inoculations were made with a 3-inch 16-gauge Ingall cannula, to the proximal end of which was attached a Fordyce handle.† The distal end of the cannula was bent over about one-quarter inch from the tip at an angle of 4° from the axis. Albino rats, weighing between 200 and 250 g, were deeply anaesthetized with ether and the cannula was inserted through the mouth into larynx and trachea. We found that with sufficient practice this could be accomplished by the sense of touch, rather than visually with the aid of a spot-light reflected into the throat, as recommended by Nungester. The degree of anesthesia was found to be highly important, since a lightly anesthetized animal showed a greater degree of spasm of the laryngeal muscles when the cannula made contact. This was not true of deeply anesthetized rats. When the tip of the cannula has entered the trachea the operator can feel the "slip" of the cartilaginous rings over the cannula's blunt, well-rounded tip. This is in contrast to the "smoothness" felt when the cannula enters the esophagus. A verification test of the position of the cannula was, however, always made by attaching to the cannula a piece of rubber tubing (fitted to an adaptor) and observing a bubble of air rising through water when the rats exhaled. After checking on the cannula's position in this manner it was gently pushed further into the trachea until greater resistance was felt at the bronchial bifurcation. At this point the handle was rotated through 90° to the right, so that the bent tip would follow into the right bronchus. Dissection of the tracheobronchial area in trial animals then revealed the tip of the cannula lying either at the entrance to the right-middle or lower lobe, or within the cardiac lobe. A previously-filled

[4] Knott, F. A., and Clark, W. H., *Lancet*, 1945, **1**, 468.

[5] Vermilye, H. N., *J. Am. Med. Assn.*, 1945, **129**, 250.

[6] Humphrey, J. H., and Joules, H., *Lancet*, 1946, **2**, 221.

[7] Southwell, N., *Lancet*, 1946, **2**, 225.

[8] Morse, F. W., *J. Am. Med. Assn.*, 1946, **132**, 272.

[9] Abramson, H. A., *An. Allergy*, 1946, **4**, 440.

[10] Barach, A. L., Silberstein, F. H., Oppenheimer, E. T., Hunter, T., and Soroka, M., *Ann. Int. Med.*, 1945, **22**, 485.

[11] Wilson, C. E., Hammond, C. W., Byrne, A. F., and Bliss, E. A., *Bull. Johns Hopkins Hosp.*, 1945, **77**, 411.

[12] Hadley, F. P., Hadley, P., McIlroy, A. P., and Laurent, A. M., *J. Bact.*, 1946, **51**, 612 (abstract).

[13] Nungester, W. J., and Jourdonais, L. F., *J. Bact.*, 1935, **29**, **34**, and *Science*, 1935, **81**, 74.

† Supplied through the courtesy of Becton, Dickinson and Company, Rutherford, N. J.

tuberculin syringe, containing the proper inoculum and with adjustment for the air volume already present in the cannula itself, was attached to the cannula base and the injection of mucin-suspended pneumococci completed. The rats tolerated this manipulation well, showing only a few minutes of dyspnea following inoculation.

A Type I strain of pneumococcus ("Bailey" strain, obtained from Dr. Eleanor Bliss) was used in all experiments. The culture was maintained in beef-infusion broth containing 5% rabbit blood and 0.1% agar. It was passed through a mouse and re-isolated on the day preceding each major experiment. Frequent tests of virulence in mice revealed a constant M.L.D. equivalent to a dosage of 10^{-8} cc (2 to 4 organisms per cc), injected intraperitoneally. However, approximately 600 organisms, suspended in mucin, were necessary to kill rats when inoculated by the intrabronchial route. In the following experiments intrabronchial inoculations were made with 60,000 mucin-suspended organisms, or 100 M.L.D. by this route (0.15 cc of a 10^{-3} dilution of a 16 hour blood broth culture). The mucin used was a 6% aqueous suspension of Wilson's granular mucin, Type 1701 W, autoclaved at 15 pounds for 15 minutes and then adjusted to pH 7.3.[14]

Under the conditions outlined above typical lobar pneumonia resulted in the untreated rats within 18 hours of inoculation. This was accompanied by septicemia and consolidation of the inoculated lobe, and a high percentage of mortality resulted. For example, in one experiment in which 30 rats were inoculated intrabronchially with mucin-suspended pneumococci, 100% of the animals died with an average survival time of 42 hours. At autopsy all animals showed consolidation of a portion or all of the inoculated lobe, and varying degrees of congestion and edema of the remaining lobes. Sections revealed acute pneumonitis, with congestion, edema, fibrin and leucocytic infiltration of the alveoli. One of these 30 animals showed fibrinous pleurisy, a condition which we found to occur more frequently in those rats which survived longer than 48 hours.

Method of Producing and Administering Penicillin Aerosol. For each experiment 20 rats were usually employed, all of which were inoculated as described. Ten were then given treatment and 10 used as untreated controls. The time after inoculation at which treatments were begun, as well as the frequency of treatments, varied in different experiments.

The rats to be treated were placed together in a wooden box, 8 x 16 x 16 inches, having a capacity of about 30 liters of air. The box was covered by a loosely-fitting glass plate through a hole in which a rubber tube was inserted as an air vent. The tip of the Vaponephrin nebulizer entered an opening at one side of the box 1½ inches below the top, while the lower end of the nebulizer was attached by a rubber connection to the L-14 oxygen flowmeter which measured the air flow, in liters per minute, through the nebulizer. This in turn was attached to an air pressure gauge and to the air pressure line. The measured penicillin solution (sodium or calcium penicillin, Pfizer), usually in a concentration of 40,000 units per cc of saline, was placed in the nebulizer as needed. Before the rats were admitted to the chamber the penicillin was sprayed into it for 5 minutes at an air-flow rate of 4 liters per minute. The chamber was thereby filled with the nebulin to such an extent that a heavy fog was visible throughout the box and could be seen escaping through the airvent. This was taken as a good indication of the rapid dissemination of the nebulin through the treatment chamber itself. Treatment periods consisted of 15 minute exposures. After the first minute or 2 the rats were at rest and appeared to be little disturbed by the effects of inhaling the penicillin mist.

Estimation of amount of penicillin inhaled by rats. The approximate number of units of penicillin inhaled by each rat was estimated by multiplying the tidal air for rats,‡ in cc

[14] Miller, C. P., and Castles, R., *J. Infect. Dis.*, 1936, **58**, 263.

‡ Formula of Navy Med. Res. Unit No. 115 for tidal air of albino rats:

$V = 212\ W^{3/4}$ where V is the volume of air inhaled, in cc per minute, and W is the weight of rat in kg.

TABLE I.
Prophylactic Effect of Penicillin.
(Administered previous to intrabronchial inoculation of pneumococci.)

Menstruum for organisms	Treatment: Route of administr.	Treatment: Total units (estimated)	Results: No. of rats	Results: No. survived	Results: % survival	Results: Avg survival time (hr)
Mucin	Inhalation	5600 to 7600	20	1	5	56
	Intramusc.	5000	10	0	0	41
	Controls	0	30	0	0	42
Broth	Inhalation	3600 to 4600	20	19	95	40
	Intramusc.	5000	10	10	100	—
	Controls	0	30	3	10	42

per minute, by the number of drug units in each cc of air leaving the atomizer (estimated from the air and fluid delivery of the atomizer). Thus the *estimated* inhalational dose of penicillin for a 200 g rat (tidal air of 63 cc/min.), during a 15 minute exposure to a mist produced from a solution of 40,000 units/cc, with an air-flow through the atomizer of 4 liters per minute, was approximately 1500 units (0.16 cc of penicillin was nebulized per minute).

We recognize that this estimation can give only an approximation of the actual dosage, since it is based on two assumptions,—first, that after the preliminary "saturation" of the chamber, the concentration of nebulin within the chamber maintained a fairly constant equilibrium, and that this concentration was the same as that of the nebulin upon leaving the nozzle of the atomizer. Considering the small volume of the chamber, and the constant flow of nebulin through the chamber and out the vent, the concentration in the chamber is believed not to have varied sufficiently to disturb the results. The *second* assumption was that the full amount of penicillin inhaled was retained in the lungs. We know this cannot be true but it is not possible at the present time to estimate that fraction which was again exhaled. Lyons and coworkers[16] have reported an experiment in which only about 20% of inhaled radioactive chromic phosphate was retained in the lungs of a monkey exposed to a mist of this substance for 40 minutes. With a variable of this order it is impossible at this time to present for our own experiments more accurate figures on the inhalational dosage. It seems certain, however, that our *estimated* drug dosage is in excess of the amount actually received and retained by the rats. Therefore, in evaluating the therapeutic effect of penicillin aerosol on pneumococcus pneumonia in rats the actual amount of the drug which gave protection or resulted in cure was less than our figures indicate.

Results. Prophylactic Effect of Penicillin on Pneumococcus Pneumonia. It was considered desirable to learn whether penicillin would prevent the development of pneumonia, when administered by either the inhalational or the parenteral route *previous to* the intrabronchial inoculation of pneumococci. A comparison was also desirable between the effect of such treatment on animals inoculated with mucin-suspended and broth-suspended organisms. The rats receiving treatment by the inhalational route were exposed to penicillin aerosol for two 15-minute periods at 4 hours and at one hour previous to the inoculation of pneumococci. The parenterally-treated rats received, by intramuscular injection, 2500 units of penicillin at each of the same two periods. The results are shown in Table I.

Of 20 rats receiving mucin-suspended pneumococci following exposure to penicillin by inhalation, only one survived. Ten additional rats in the same group, which received penicillin intramuscularly, showed no survivals. All 30 control untreated rats died. Thus, neither the inhalational nor the parenteral adminis-

[15] Personnel of U. S. Navy Med. Res. Unit No. 1, and Kleiber, M., *Science*, 1944, **99**, 542.

[16] Personnel of Naval Lab. Res. Unit No. 1, and Lyons, W. R., *Am. J. Med. Sc.*, 1944, **207**, 40.

tration of penicillin, previous to the inoculation of mucin-suspended pneumococci, prevented death from pneumonia. The average survival time (56 hours) of the group receiving inhalations was somewhat higher than that of the parenterally-treated group (41 hours) and the controls (42 hours).

On the other hand, when broth-suspended organisms were inoculated, following similar administration of the drug, the survival rates were high. In the group receiving inhalational therapy 95% survived; of those receiving intramuscular injections 100% survived, as compared with 10% survival among the controls.

The mechanism by which a prophylactic dose of penicillin, given by inhalation or parenterally, prevents infection by pneumococci suspended in broth, while it does not prevent infection by organisms suspended in mucin, is not clear. Untreated control animals in both groups showed similar high fatality rates (100% for the mucin group, 90% for the broth group), as well as the same average survival time (42 hours). It is true, as Nungester[17] has pointed out, that broth-suspended pneumococci, injected intrabronchially, cause a pulmonary infection showing less consolidation than when mucin-suspended organisms are injected. In harmony with this observation we found that of 30 control rats injected with broth suspensions approximately 50% showed, at death, only a severe pulmonary congestion, while about 50% showed some degree of consolidation, 20% of the latter group involving more than half of one lobe, and 30% involving less than half. On the other hand, among the 30 control rats receiving mucin suspensions, all showed some degree of consolidation at death, 83% showing more than half of one lobe consolidated. All animals showed septicemia at death.

One possible explanation of the failure of survival of animals receiving mucin-suspended organisms following prophylactic administration of penicillin is that the mucin formed around the organisms a protective barrier against penetration by the penicillin. On the other hand, a chemically-inhibiting effect of mucin upon small amounts of penicillin is not out of the question, although a preliminary experiment carried out *in vitro* indicated that mucin has no inhibiting effect upon penicillin. We have also verified Nungester's observation that mucin does not support the growth of pneumococci, *in vitro*. We believe that, until further study has been conducted, it is not possible to reach a conclusion on the causes for the difference in effect of penicillin (given prophylactically) on broth suspensions and mucin suspensions of pneumococci.

Therapeutic Effect of Penicillin Inhalations. In these experiments penicillin inhalations were given at various intervals following intrabronchial inoculation of *mucin-suspended* pneumococci. In Table II are presented 2 groups of experiments in which comparisons are made between the therapeutic effects of penicillin inhalations begun soon after inoculation, and those begun after a delay of 18 hours. In the first group (designated "Early" in the table) it may be noted that when 4 exposures to penicillin aerosol (each of 15 minute duration) were given, at 1, 3, 5 and 7 hours after inoculation 22 (73%) of the 30 rats so treated survived. When 8 treatment periods were given at 1, 3, 5 and 7 hours, and at 22, 24, 26 and 28 hours following inoculation, 100% of 20 rats survived. These survival rates are to be contrasted with those of the control untreated group (controls for all of these experiments are combined in the table) in which 9% of 78 rats survived.

In the "delayed" treatment group the first treatment was not given until 18 hours after inoculation. We had shown previously in untreated rats sacrificed at 18 hours, that consolidation of the inoculated lobe had already begun by this time, and septicemia had taken place. Treatment started at this time was therefore a severe test of the therapeutic effectiveness of the drug. Five treatments at 2-hour intervals beginning 18 hours after inoculation resulted in the survival of 69% of 16 rats; and 10 treatments at intervals from 18 to 72 hours caused a 70% survival

[17] Nungester, W. J., and Jourdonais, L. F., *J. Infect. Dis.*, 1936, **59**, 258.

TABLE II.
Therapeutic Effect of Penicillin Inhalations.
(Administered after intrabronchial inoculation of mucin-suspended pneumococci.)

	Treatments						
Group	No.	Hr after infection	Total estimated units	No. of rats	No. survived	% survival	Avg survival time (hr)
arly	4	1-3-5-7	5,000	30	22	73	73
	8	1-3-5-7; 22-24-26-28	12,000	20	20	100	
elayed	5	18-20-22-24-26	7,500	16	11	69	85
	10	18 to 72	16,750	20	14	70	58
ontrol	0		0	78	7	9	48

mong 20 rats. The survival time for those eated rats which died was in all cases higher ian that for the fatal control cases, as seen i Table II.

These results indicate that penicillin, administered solely by inhalation of the aerolized solution, has a definite therapeutic ffect upon pneumococcus pneumonia in rats. n these experiments, approximately 70% of ie animals were saved by either one series f four 2-hourly treatments, beginning one our after inoculation; or by one series of ve 2-hourly treatments, beginning 18 hours fter inoculation. And 100% of animals in 2 experiments involving 10 rats each) ere saved by a combination of early and te treatments, *viz.*, four 2-hourly treatments eginning one hour after inoculation, followed y four 2-hourly treatments beginning 22 ours after inoculation. Only 9% of 78 ontrol untreated rats survived.

Summary. 1. The prophylactic effect of enicillin, whether administered by inhalation parenterally, upon experimental pneumoccus pneumonia in rats, was negligible if ie infection (intrabronchial) was produced ith pneumococci suspended in mucin. If, owever, the organisms were suspended in oth the prophylactic effect was such as to determine a 100% survival rate.

2. When penicillin inhalations were given at intervals of 1, 3, 5 and 7 hours following intrabronchial infection with 100 M.L.D. of mucin-suspended pneumococci, a 73% survival rate resulted (9% for untreated controls) and the survival time of all treated rats which died was lengthened compared with that of the controls.

3. If the penicillin treatment by inhalation was delayed for 18 hours after infection (permitting the establishment of lung consolidation and septicemia), and then given from the 18th to the 26th hour, at 2-hourly intervals, the survival rate of the treated animals was 69%.

4. When penicillin inhalations were given in 2 series of treatments, at 2-hourly intervals from the 1st to the 7th hour after infection and again at 2-hourly intervals from the 22nd to the 28th hour, 100% of the animals survived compared with a 100% fatality rate for controls in this experiment.

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Effect of Streptomycin Aerosol on Friedlander's Pneumonia in Rats.*

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In view of the demonstration of the effectiveness of penicillin aerosol on experimental pneumococcus pneumonia in rats, reported previously by us,[1] it became of interest to ascertain the effect of streptomycin inhalations on an experimental pulmonary infection produced by gram-negative organisms. Favorable results following the use of streptomycin aerosol in human pulmonary infection have been reported by Olsen[2] in 9 cases of bronchiectasis, in the sputum from which gram-negative bacteria predominated; and by Menefee and Fogel[3] in 2 cases of Friedländer's infection. Human cases of Friedländer's pneumonia are rare, but very severe and nearly always fatal. Friedländer's bacillus (*Klebsiella pneumoniae*) is known to be sensitive to streptomycin. In experiments on mice infected intranasally with this organism Heilman[4] has shown that streptomycin, administered subcutaneously every 12 hours, beginning three hours after infection, had little effect in protecting them when the treatment covered a 3 day period, but that this dosage gave definite protection when treatment was continued for 7 days.

Since albino rats are known to be susceptible to infection with Friedländer's bacillus an experimental approach to the problem of the effectiveness of streptomycin inhalations on Friedländer's pneumonia was available, similar to that used in our pneumococcus study in which intrabronchially-infected rats were treated by exposure to penicillin-containing mist. The present report gives the results of 4 experiments in which rats, infected intrabronchially with mucin suspensions of Friedländer's bacillus, were treated successfully with inhalations of streptomycin aerosol. In two experiments comparison was made between the effect of intramuscular and inhalational administration of streptomycin.

Methods. Infection of rats. Rats weighing from 175 to 250 g were used. Through the courtesy of Dr. F. R. Heilman of the Mayo Clinic a Type A strain No. 837 of the Friedländer bacillus in the mucoid phase was obtained. This was highly virulent for mice in dilutions up to 10^{-6} cc by the intraperitoneal route and was found to be sensitive to streptomycin in a concentration of 0.5 μg/cc.

Preliminary intrabronchial inoculations of rats, by the technique previously described,[1] showed the lethal dose by this route to be 300 organisms (0.15 cc of a 10^{-7} dilution of a 16-hour broth culture) when suspended in 6% mucin. Intrabronchial doses of 50,000,000 organisms, suspended in *broth* did not cause death. The survival time after one lethal dose of 300 organisms, in mucin was approximately 150 hours. With large doses the survival time decreased, 30,000,000 mucin-suspended organisms (100,000 M.L.D.) giving an average survival time of 50 hours. In the treatment experiments the dosage of mucin-suspended organisms ranged from 5,000,000 (17,000 M.L.D.) to 30,000,000 (100,000 M.L.D.).

Rats infected intrabronchially and dying from Friedländer pneumonia showed pulmonary lesions similar to those observed in the disease in man. The inoculated lobe was

* Work done under a grant from the Medical Division of the Chemical Warfare Service, Edgewood Arsenal, Md.

1 Hadley, F. P., McIlroy, A. P., and Laurent, A. M., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 204.

2 Olsen, A. M., *Proc. Mayo Clinic*, 1946, **21**, 53.

3 Menefee, E. E., and Fogel, D. H., *N. Carolina Med. J.*, 1947, **8**, 165.

4 Heilman, F. R., *Proc. Mayo Clinic*, 1945, **20**, 33.

reatly enlarged, light gray in color, and con-olidated. Sections showed edema, acute neumonitis, with necrotic areas. The other obes showed an acute inflammation. All obes and the heart were covered with a ick, slimy exudate, containing encapsulated rganisms. Occasional rats developed lung bscess, with adhesions to the diaphragm and hest wall.

Treatment. The first treatment was given ithin 1 or 2 hours after intrabronchial in-culation of mucin-suspended organisms. For nhalational treatment 10 rats were placed ogether in the treatment chamber[1] into hich the streptomycin-containing mist was ntroduced from a *Vaponephrin* nebulizer con-aining an 0.85% NaCl solution of the drug, n a concentration of 50,000 or 75,000 μg/cc. he apparatus and the method of calculating ne amount of streptomycin inhaled by each at were described in a previous report.[1] The verage *estimated* amount of the drug inhaled y each rat in the different experiments anged from 65 to 150 μg per minute, or a otal of from 1,000 to 2,200 μg per 15 min-tes of treatment. As in the penicillin aerosol xperiments the *actual* dosage by the inhala-onal route was probably much lower than ur calculated figures. The reasons for this re explained in the previous paper.

Inhalational treatment began 1 or 2 hours fter infection, and continued on the first ay at 3-hour intervals until 4 treatments ad been given. Each treatment consisted f a 15-minute exposure to the mist. This hedule was followed on 3 or 4 successive ays.

Rats receiving streptomycin by intramus-ular injection were treated at the same in-rvals with 1,000 μg dissolved in 0.1 cc of hysiological saline in Experiment I and ith 500 μg in Experiment IV.

Results. Table I summarizes the results the experiments. In Experiment 1 30 ts were inoculated intrabronchially with),000,000 (100,000 M.L.D.) mucin-sus-ended organisms. Beginning one hour after fection 10 rats received in a 3-day period total of 11 intramuscular injections of 1,000 of the drug. All of these rats survived.

TABLE I.

Effect of Streptomycin, Administered Parenterally and by Inhalation, on Friedlander Pneumonia in Rats.

Exp. No.	No. of organisms inoculated	Treatment					Results		
		Route	No.	Days	Hr after infection	Total μg per rat × 1000	No. of rats	No. survived	% survival	Avg. surv. time (hr)
I	30,000,000 (100,000 M.L.D.)	Intramuse.	11	3	1 to 54	11	10	10	100	—
		Inhalation*	11	3	1 to 54	11	10	6	60	103
		Control	—	—	—	—	10	0	0	50
II	28,000,000	Inhalation*	15	4	1 to 77	15	10	7	70	78
		Control	—	—	—	—	10	0	0	54
III	8,000,000 (30,000 M.L.D.)	Inhalation†	16	4	1 to 78	25.6	10	7	70	128
		Control	—	—	—	—	10	0	0	69
		Intramuse.	16	4	2 to 79	8	5	5	100	—
IV	5,000,000 (17,000 M.L.D.)	Inhalation†	16	4	2 to 79	35	10	10	100	—
		Control	—	—	—	—	10	2	20	95

* 50,000 μg/cc salt solution. † 75,000 μg/cc salt solution.

In addition, 10 rats received 11 inhalational treatments with streptomycin aerosol over a 3-day period (estimated 1,000 μg per treatment). Six of these rats survived. All 10 untreated rats died from pneumonia in an average time of 50 hours. The average survival time of the 4 rats which died, despite inhalational treatment, was 103 hours.

In Experiments II and III, in which the infective dose was 28,000,000 and 8,000,000 organisms respectively (100,000 and 30,000 M.L.D.), 4 inhalational treatments per day for 4 days resulted in a 70% survival, with no survivals among the controls.

In Experiment IV a comparison was again made between parenteral and inhalational therapy following the intrabronchial inoculation of 5,000,000 organisms (17,000 M.L.D.). The 5 rats receiving 16 intramuscular injections of 500 μg each (total of 8,000 μg) over a 4-day period showed a 100% survival. Ten rats receiving 16 inhalational treatments with an estimated 2,200 μg per treatment (total of 35,000 μg), also showed a 100% survival. Of the 10 untreated control rats, 2 survived. The 2 surviving controls showed, when sacrificed at 14 days, lung lesions consisting, in one case, of an abscess containing viable Friedländer's bacilli; and in the other rat consolidation of the right lower lobe, with adhesions; whereas all treated rats, when sacrificed at 14 days, showed no lesions of any kind.

It is apparent from these experiments that the intramuscular route of administration of streptomycin was more effective in controlling an *overwhelming* dosage of Friedländer's bacilli (100,000 M.L.D.) than was the inhalational route. Moreover, that a 100% survival rate following inhalational treatment was possible only when the infective dosage of organisms was decreased to 5,000,000 (17,000 M.L.D.). And even at this dosage a smaller amount of the drug gave protection when administered intramuscularly than when it was given by the inhalational route (8,000 μg and 35,000 μg respectively), although the actual inhalational dose was probably less than half the estimated one.[1]

A possible explanation for the greater effectiveness of the intramuscular mode of administration, especially under conditions of tremendous infective dosages, can be found in the circumstance that a septicemia soon takes place, as is well known to occur with any type of Friedländer infection in rats or mice, and that this septicemia naturally is controlled more quickly by parenteral administration than by inhalational. This view receives further support from our observations[5] that streptomycin in contrast to penicillin, is poorly absorbed from the lung into the blood stream.

Summary. Streptomycin, administered to white rats by intramuscular injection or by inhalation, *following* intrabronchial inoculation of mucin-suspended Friedländer's bacilli, is highly effective in preventing a consequent pneumonia. Of 10 rats infected with 100,000 lethal doses and treated by intramuscular injection of 1,000 μg of streptomycin 4 times a day for 3 days, all survived, while 10 control, untreated rats died within 2 days. Similarly, injections of 500 μg 4 times a day for 4 days saved all of 5 rats inoculated with 17,000 lethal doses, while 8 of 10 control rats died within 4 days.

When the drug was given at similar intervals by inhalation of streptomycin aerosol to 30 rats infected intrabronchially with from 30,000 to 100,000 lethal doses, 60 to 70% of the treated animals survived following estimated dosages of from 1,000 to 1,600 μg per treatment. All of the 30 untreated control rats died. To the foregoing data it may be added that, when the dosage was reduced to 5,000,000 organisms, which constituted a dose of 17,000 M.L.D., and inhalational treatment was given at the rate of an estimated 2,200 μg per 15-minute treatment 4 times a day for 4 days, 100% of 10 treated rats survived, while 80% of 10 controls died within 4 days.

Streptomycin (sulfate) used in this study was generously supplied by Chas. Pfizer and Company, Brooklyn, N. Y.

[5] Laurent, A. M., McIlroy, A. P., and Hadley, F. P., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 213.

16436

Penicillin and Streptomycin in Lungs and Blood Serum of Rats Following Inhalational and Intramuscular Administration.*

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(Introduced by Philip Hadley.)

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In connection with a study of the therapeutic effects of penicillin and streptomycin aerosols on experimental pneumococcus and Friedländer's pneumonia in rats,[1,2] it became of interest to ascertain the concentration of these drugs in lungs and blood serum of normal rats at various times following administration by the inhalational and intramuscular routes.

Little work has been reported on the detection of these drugs in the tissues of animals. Bryson, Sansome and Laskin[3] recovered a "bacteriostatic substance" from the lungs and urine of a rabbit exposed to penicillin aerosol for 2 hours; also from the lungs of 4 mice exposed for 1 hour. Kornegay, Forgacs and Henley[4] reported that in guinea pigs streptomycin was absent in lung, spleen and liver extracts following parenteral administration of this drug.

Blood levels of these drugs have been carefully studied by many workers, particularly following parenteral administration. Zintel and co-workers[5] have shown that streptomycin is detectable in the blood for 6 hours following a single intravenous injection as compared with 2½ to 3 hours in the case of penicillin. Wilson, Hammond, Byrne and Bliss[6] found that rats given penicillin by the inhalational route showed a higher blood level within a few minutes than those animals receiving the drug by the intramuscular or oral route; also, that the drug was maintained longer in the blood following inhalation.

Methods. Drug Dosage. Ten normal, albino rats weighing approximately 250 g were chosen for each experimental group. In the *penicillin* experiments those receiving the drug parenterally were given a single intramuscular injection of either 2,000 or 5,000 units of calcium penicillin (Pfizer) in 0.85% salt solution. The rats receiving penicillin by inhalation were exposed for 15 minutes to nebulized penicillin produced from a saline solution containing 40,000 units per cc. Approximately 2,000 units were estimated to have been inhaled by each rat during this exposure, but the *actual* retained inhalational dose was probably much less. The technic used in administering the aerosol and the method of calculating the inhalational dose were described in a previous report.[1]

In the *streptomycin* experiments intramuscular injections of either 2,500 or 5,000 μg of streptomycin sulfate (Pfizer) were given. Rats receiving the drug by inhalation were exposed for 15 minutes to a mist produced by nebulizing a solution of streptomycin containing 75,000 μg per cc of salt solution. The average *estimated* inhalational dose per rat was approximately 2,300 μg.

Preparation of Lung Extract. In each ex-

* Work done under a grant from the Medical Division of the Chemical Warfare Service, Edgewood Arsenal, Md.

1 Hadley, F. P., McIlroy, A. P., and Laurent, A. M., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 204.

2 Hadley, F. P., Laurent, A. M., and Onslow, J. M., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 210.

3 Bryson, V., Sansome, E., and Laskin, S., *Science*, 1944, **100**, 33.

4 Kornegay, G. B., Forgacs, J., and Henley, T. F., *J. Lab. and Clin. Med.*, 1946, **31**, 523.

5 Zintel, H. A., Flippin, H. F., Nichols, A. C., Wiley, M. M., and Rhoads, J. E., *Am. J. Med. Sc.*, 1945, **210**, 421.

6 Wilson, C. E., Hammond, C. W., Byrne, A. F., and Bliss, E. A., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 451.

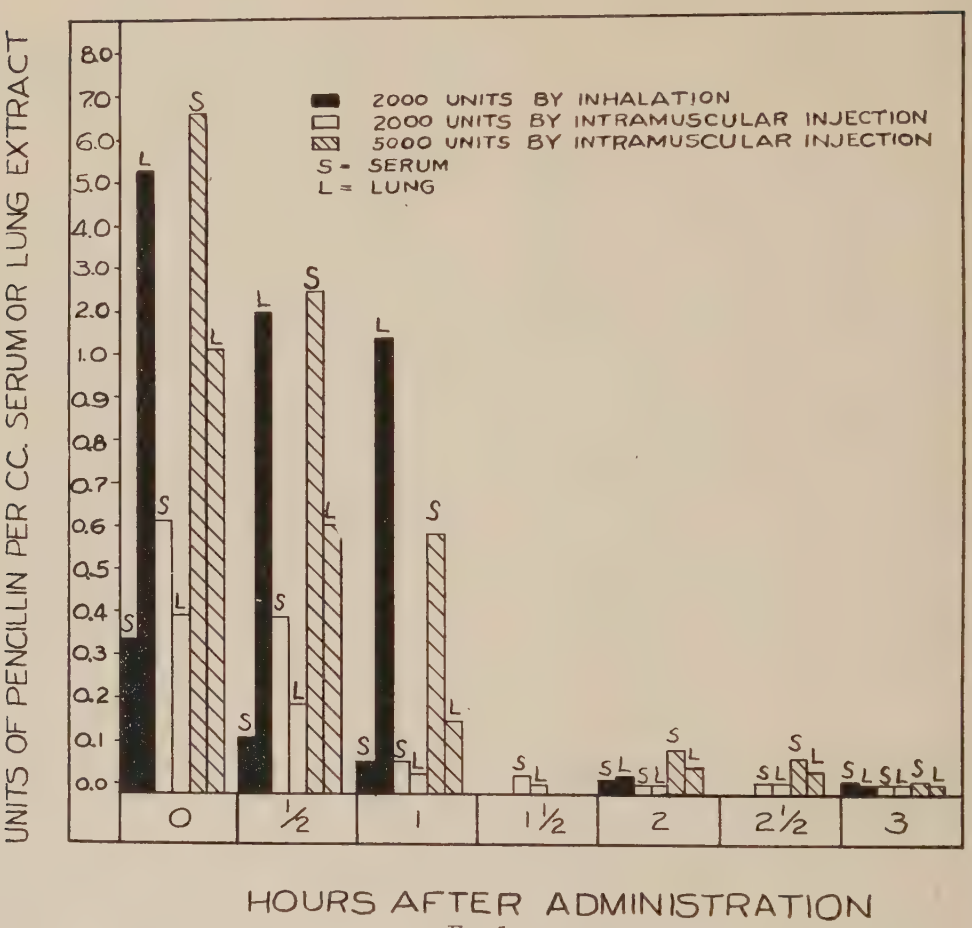

FIG. 1.
Penicillin levels in rat serum and lungs.

periment 2 rats were sacrificed at times ranging from a few minutes to 3 hours after administration of the drugs. Under ether anesthesia each rat was exsanguinated by the removal aseptically of 6 to 9 cc of blood from the heart, followed by the severance of blood vessels in the neck to permit further bleeding. The lungs were removed aseptically *in toto*, and each lobe, dissected away from the trachea, was blotted on sterile filter paper to remove traces of blood adhering to the surface. They were then ground with sand and 1.5 cc of salt solution and the resulting paste transferred to tubes and centrifuged for 5 minutes at about 5,000 R.P.M. The supernatant material was removed and stored in the icebox, together with the blood serum, until drug concentrations were determined on the following day. Separate determinations were made for each animal.

Methods of Assay. Penicillin in lung extracts and serums was determined by the method of Rammelkamp[7] using a 16 hour broth culture of the C203M strain of hemolytic streptococcus. Veal-infusion broth (pH 7.4) was used to dilute samples and penicillin standard.

Streptomycin was determined by the method of Donovick and Rake[8] with some modifications. A 6-hour broth culture of

[7] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

[8] Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

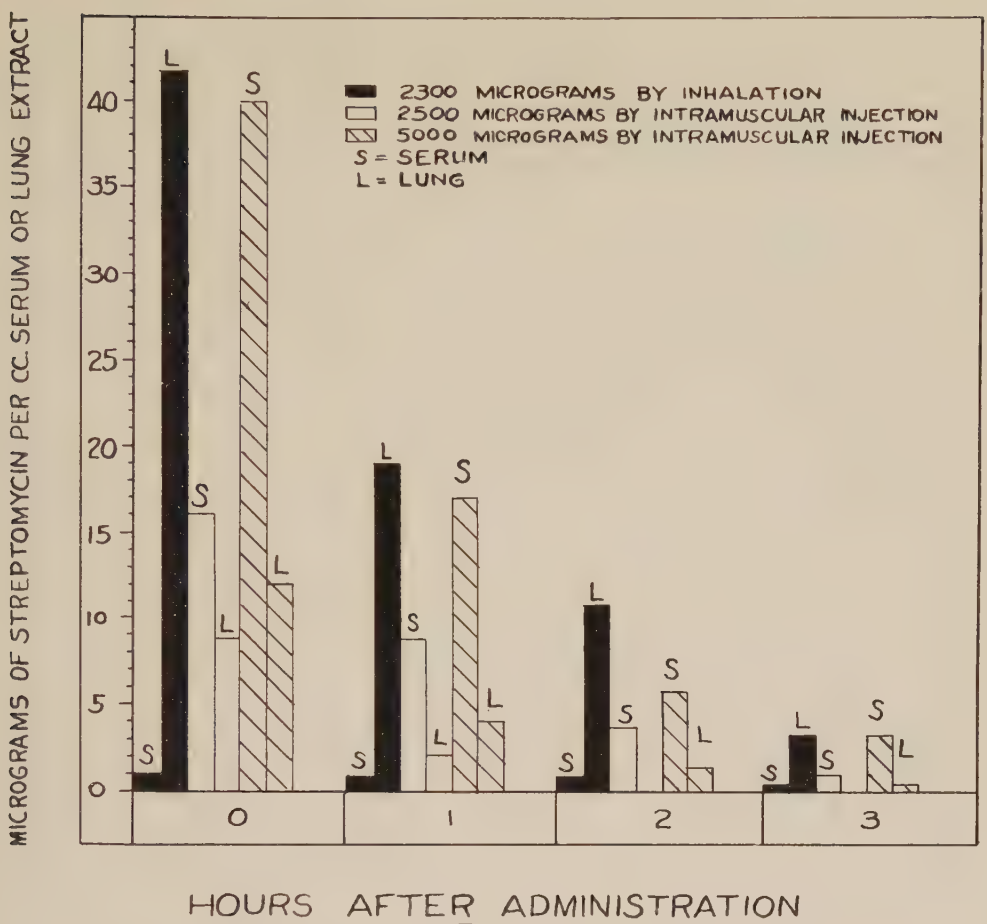

FIG. 2.
Streptomycin levels in rat serum and lungs.

Friedländer's bacillus (No. 837) was the test organism, and Difco yeast broth (pH 7.0) was used to dilute the streptomycin standard and samples.

Results. Penicillin Concentrations. Fig. 1 presents the summarized data for several experiments. The solid bars, representing units of penicillin per cc of serum or lung extract following inhalation, show the average concentration for 10 rats after each time interval. The striped and open bars, representing concentrations following intramuscular injection, show average figures for 2 rats after each time interval.

Lungs. It may be seen from the graph that the lungs contained a much higher concentration of penicillin (5.3 units/cc lung extract) immediately (or within the first few minutes) following a 2,000 unit inhalational dose than following either 2,000 or 5,000 units by injection (0.39 and 1.17 units/cc lung extract, respectively). This was true also at one-half and 1 hour intervals. It is noteworthy that the high lung concentration following 2,000 units by inhalation persisted for at least 1 hour, at which time it was 60 times greater than that resulting from a 2,000 unit intramuscular dose, and 10 times greater than that following a 5,000 unit intramuscular dose. At 2 hours penicillin was still detectable in the lungs following inhalation, but it was absent at this time following a similar intramuscular dose. At 3 hours it was absent in all lung samples.

Serum. Comparing the serum concentrations following the 3 methods of administra-

tion it may be seen that a 2,000 unit inhalational dose resulted immediately in an effective level (0.33 units/cc) which dropped to 0.04 units/cc at 1 hour. A 2,000 unit intramuscular dose resulted in a serum level twice as high immediately (0.6 units/cc) but at 1 hour it equaled the serum concentration attained at this period following inhalation. The 5,000 unit intramuscular dose gave the highest serum levels which were maintained at inhibitory concentrations through 2½ hours.

Streptomycin Concentrations. In Fig. 2 are presented figures for streptomycin levels in serum and lungs at various times following administration of the following doses: 2,300 μg by inhalation, 2,500 μg and 5,000 μg by intramuscular injection. Each bar in the diagram represents the average value obtained from determinations made on 2 rats (in 70% of the assays the values were identical for the 2 animals).

Lungs. Immediately following inhalation of 2,300 μg of streptomycin the concentration in the lungs was 41.6 μg/cc of lung extract, whereas intramuscular injections of 2,500 and 5,000 μg resulted in immediate concentrations of only 8.7 and 11.6 μg, respectively. At 1 hour following inhalation the lung concentration was 18.7 μg, while only 2 and 4 μg, respectively, were detected following injection of 2,500 and 5,000 μg. Even at 2 and 3 hours after inhalation the lungs contained 10 μg and 3 μg, respectively, although none was detectable at these times following a similar dose by injection. At 2 and 3 hours following a 5,000 μg injection 1.2 and 0.4 μg, respectively, were found.

Serum. It is to be noted that the concentration of streptomycin in the serum following the 2,300 μg inhalational dose remained at less than 1 μg/cc at all 4 time intervals. This indicates that, in spite of the high lung concentration, this drug is poorly absorbed into the blood from the lungs. A 2,500 μg injection resulted in serum levels ranging from 15 μg/cc immediately to 1 μg at 3 hours. And following a 5,000 μg injection serum levels ranged from 40 μg/cc immediately to 3 μg at 3 hours.

Summary. 1. Penicillin and streptomycin were found in the lungs of rats in far greater concentrations following inhalational administration than after intramuscular injection of similar and even higher doses.

2. Penicillin was retained in the lungs in a high concentration for 1 hour following inhalation. It was still detectable in smaller amounts at 2 hours.

3. Streptomycin was retained in the lungs in an effective concentration for at least 3 hours following inhalation.

4. Penicillin was present in the blood stream in effective concentrations for 2 hours following inhalation.

5. Streptomycin was poorly absorbed from the lungs into the blood following inhalation.

Penicillin and streptomycin used in this study were generously supplied by Chas. Pfizer and Company, Brooklyn, N. Y.

16437

Effect of Infused Streptomycin in the Mammary Gland.*

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With the establishment of streptomycin as a potent chemo-therapeutic agent in the treatment of many infections resistant to either sulfonamides or penicillin the possibility of its use in the treatment of some forms of infectious bovine mastitis became apparent. This present study was undertaken to deter-

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TABLE I.
Records of Amounts of Streptomycin Infused and Recovered in the Milk with Varying Time Intervals and Quantities of Milk Secreted.

Animal No.	Quarter infused	Streptomycin infused, units × 1000	Time between infusion and milking hr	Total am't of milk obtained at milking ml	Concentration per ml milk units	Amount recovered units ×1000
3	R.R.	500	24	2450	172	421.4
3	L.F.	300	24	1750	100	175
3	R.R.	200	24	2400	50	120
1	R.R.	200	24	2875	49	140.888
3	L.F.	200	24	1100	73	80.3
3	R.F.	200	24	1100	70	77
3	L.F.	200	16	1000	112	112
3	R.F.	200	12	1000	72	72
3	L.R.	200	40	1400	75	105
659	R.F.	150	24	2320	40	92
763	R.F.	150	24	4165	20	82
659	R.R.	150	12	1150	67	77
763	R.R.	150	12	1680	42	70.5
841	R.R.	100	12	970	47-64	—
3	L.R.	100	24	1000	63	63
841	R.F.	100	12	570	80-116	—

TABLE II.
Concentration of Streptomycin in Successive Milkings Following Infusion and Proportion of the Infused Drug Recovered.

Animal No.	Quarter infused	No. of units/ml ×1000	Units per ml of milk at various milking intervals following infusion of quarter					Total recovery in %
			12 hr	24 hr	32 hr	48 hr	72 hr	
763	R.R.	150	42	14	—	—	—	—
3	L.F.	200	—	73	50	18	8	41
3	L.R.	200	—	80	64	30	15	62
3	R.F.	200	72	28	25	7	—	50
3	R.R.	200	50	21	17	—	—	64
3	R.R.	500	—	172	56	30	None	92
3	L.F.	300	—	100	80	30	"	104
3	L.R.	100	—	63	40	17	"	76
3	R.F.	200	—	70	20+	20+	—	—
3	R.R.	200	—	64	20+	20+	—	—

mine the pharmacological properties of the antibiotic when infused into the non-infected gland. Points of major interest included the relationship of size of dose, to concentration in the gland, maintenance of concentration, manner of elimination from the gland, and toxicity.

Materials and Methods. Five grade Holstein cattle and one grade Togenberg goat were employed. All animals were lactating normally and were free of disease during this study. The history and physical examination of the gland of one cow indicated the presence of disease at some previous time.

The cup-plate method of assay slightly modified as reported by Beadle, Mitchell and Bonner[1] and adapted to measurement of antibiotic in milk by Weirether, Jasper and Peterson[2] was used for determining the concentration of streptomycin in the milk samples. Notable differences from the latter were, the agar used, the test organisms, and the procedure of handling the assay plates after their preparation. The agar was the commercial Streptomycin Assay Agar. Originally the test organism was a strain of *Staphylococcus aureus* isolated at the University of Minnesota which later was replaced by a culture of *Staphylococcus aureus, S. M.*

[1] Beadle, G. W., Mitchel, H. K., and Bonner, D., *Bact.*, 1945, **49**, 101.

[2] Weirether, V. J., Jasper, D. E., and Petersen, W. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 282.

After preparation the assay plates were handled in the usual manner for streptomycin cup-plate procedures. Herrel and Heilman.[3]

Procedure. Milk samples were obtained by milking out the quarter completely and retaining a small portion of the whole for assay. The interval between infusion of a quarter with streptomycin and evacuation of the milk for assay varied from 12 to 40 hours. In some instances a quarter was milked out completely several times; a sample being retained each time for assay. The interval between successive milkings varied from 3 to 24 hours and between the first and last milking from 12 to 72 hours. Samples were either assayed immediately or refrigerated until assay could be performed.

Assay of urine samples was made in some cases. Urine samples from the cow were obtained by artificial stimulation of the micturation reflex. Samples from the goat were secured by catheterization. Following an infusion, 5 to 11 samples were obtained over a period of 24 to 32 hours.

Blood samples following infusion of the mammary gland in the goat were obtained for assay in two instances. Over a 24-hour period 10 samples were drawn in each case. The blood was permitted to clot and the serum saved for assay.

Gross appearance of the milk, its quantity, pH, and cell and chloride contents were determined as a basis for evaluating the irritating and toxic effects of the drug on the mammary glands.

The amount of drug infused varied from 100,000 units to 500,000 units per quarter in the cow. In the goat either 140,000 or 200,000 units were infused into one-half of the udder. The streptomycin was dissolved in sterile distilled water. The concentration in the solution to be infused was uniformly maintained at 20,000 units per ml. The volume of material infused thus varied with the total number of units infused.

Table I illustrates the general relationship between concentration per ml in the sample and (1) size of dose (2) time, *i.e.*,

[3] Herrel, Wallace E., and Heilman, Fordyce R., *Am. J. Med.*, 1947, **2**, 421.

TABLE III.
Elimination of Streptomycin in the Urine at Varying Time Intervals Following Intramammary Infusions.

Animal	Amount infused ×1000	Quarter (cow) of half (goat) of udder infused	Units/ml of urine in sample drawn at various intervals (hr) following infusion															
			1	2	4	5	6	7	8	9	10	11	13	14	17	24	26	27	32
Goat	140	left	9	9	15+	—	15+	—	15+	15+	—	15+	—	15+	—	15+	—	15+	—
Goat	200	"	—	—	—	—	—	12	—	15	13	—	13	—	—	—	—	13	—
Cow	1,100	all 4	—	—	—	2	—	125	—	14	15	—	135	14	—	11.3	8	8.5	9.5

interval between infusion and sampling and (3) dilution or production of the quarter. It should be expected that these three factors would be among the more important ones in determining concentration at any given time. An interesting feature presented here is the seemingly small amount of influence a 24-hour variation of factor No. 2 had. Another notable feature is that on the dosage schedules used the concentration did not fall below 20 units / ml for periods up to 40 hours. Assays of the first and last drawn milk gave equivalent values.

The above observations promoted speculation concerning the elimination of streptomycin from the gland. In Table II are presented total recovery percentages and figures identifying the presence of streptomycin at various intervals following infusion. The data accumulated would seem to indicate that whatever the mechanism for elimination be, it must represent a relatively slow process. Also, since each milking was a complete one, in some instances aided by the intravenous injection of 10 units of oxytocin, and since successive milkings revealed considerable amounts of streptomycin, it was thought that a certain percentage might temporarily be fixed locally in the tissues. The amount so fixed, it was postulated, diminished as accumulating secretions in the gland became equilibrated with the tissues.

Among the more obvious ways by which the streptomycin unaccounted for might disappear from the normal gland are (1) destruction in the tissues and (2) absorption into the blood and elimination by the kidneys in the urine. The first named possibility was not investigated. For exploring the second the goat was first employed. In this animal the volume of blood and urine are relatively small thereby increasing the chance for detection of the antibiotic. After two such experiments on the goat a third was performed on the cow. The data are presented in Table III. The levels reached in the urine are not exceptionally high but are maintained for a considerable length of time at a fairly consistent value. Assays were not continued over a long enough time to determine the end point. However, if the normal average urine production of these animals be considered it would appear that the major portion of the antibiotic not accounted for in the milk might be excreted in the urine. Further experiments are planned to study the rate of renal excretion following infusion.

At no time was there sufficient streptomycin present in the blood to be detected by the assay methods used.

In only 3 experiments (those for cows 763, 841, and 659) were the pH, chlorides and cell counts completed for the milk before and after the infusions. No changes in the pH or chloride values were observed and only a slight increase in cell numbers which lasted for only a few milkings. No irritating effects could be detected by clinical inspection of the udders and the milk appeared normal except for a slight discoloration occasionally observed in the first milking following the infusion.

Summary. (1) Streptomycin infused into the normal bovine mammary gland in amounts ranging from 100,000 to 500,000 units per quarter could be detected in milk samples as long as 48 hours following infusion. As determined by the assay procedure used, the concentration did not fall below 20 units / ml in any of the samples after a 24-hour interval. (2) The concentration per ml in the sample was found to vary with (1) the size of the dose (2) time interval between infusion and sampling and (3) milk production of individual quarters. (3) At no time was there sufficient streptomycin present in the blood to be detected by the assay procedure used. However, in both the cow and goat significant amounts were found in urine samples as long as 27 hours following infusion. (4) Under the conditions of these experiments streptomycin was found to be relatively non-toxic when infused into the normal bovine mammary gland.

Acknowledgment is gratefully made to Dr. D. F. Green of Merck and Co., Rahway, N.J., for furnishing the streptomycin used in these experiments.

16438 P

Electron Microscopy of Sickle Cells.

J. W. Rebuck, H. L. Woods, and E. A. Monaghan.
(Introduced by F. W. Hartman.)

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Since sickle cell anemia was first described by Herrick,[1] there have been numerous descriptions of the changes in the red corpuscular structure in this disease as observed in optical studies. The corpuscles progressively transform in structure and shape from the customary discs into elongated forms curved in the mid-portion and pointed at either end, into multi-pointed forms, needlelike forms, forms resembling exaggerated crenation, oat-like forms, and other bizarre types. Many additional forms, transitional between discs and the above group collectively designated as "sickle cells," have been illustrated by Diggs and Bibb.[2]

Such changes are readily observed in sealed wet preparations of whole blood from the patient. However, "sickled" corpuscles usually revert to their rounded forms upon exposure to air. This necessitates application of a fixative to the sickled corpuscles before such exposure to air, in order to obtain permanent preparations.

Beck and Hertz[3] introduced the red corpuscles into a saline citrate solution which was then sealed with paraffin oil. After sickling had been accomplished upon standing a solution of formaldehyde was added to the blood-saline-citrate mixture and reversion to the corpuscular shape was prevented. Permanent preparations of the sickled cells were thus obtained.

For electronic studies, the Beck-Hertz technic was modified as follows. The blood of a negro patient with sickle cell anemia was employed. This patient was recovering from an acute hemolytic crisis. Blood was introduced into paraffin-lined Wassermann tubes in which had been placed a small amount of powdered heparin. The heparin-blood mixture was covered with paraffin oil and allowed to stand 21 hours. Formalin-saline was then added to the now sickled erythrocytes which were allowed to stand an additional 24 hours. The formalized sickle cells were removed from the paraffin oil-sealed tubes and washed 4 times in physiological saline solution. They were then spread over formvar-covered glass slides and dried over phosphorus pentoxide. Final direct specimen mounting was performed by the modified stripping technic previously described.[4] For a general discussion of the effects of formalin fixation upon cytoplasmic structure with electronic methods, the reader is referred to the work of Porter, Claude and Fullam.[5]

Our Fig. 1 (× 5,000) is an electron micrograph which presents one of the crescent forms

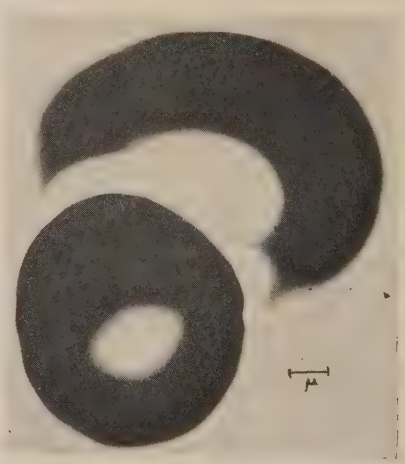

Fig. 1.

[1] Herrick, J. B., *Arch. Int. Med.*, 1910, **6**, 517.

[2] Diggs, L. W., and Bibb, J., *J.A.M.A.*, 1939, **112**, 695.

[3] Beck, J. S. P., and Hertz, C. S., *Am. J. Clin. Path.*, 1935, **5**, 325.

[4] Rebuck, J. W., and Woods, H. L., *Blood*, 1948, **3**, 175.

[5] Porter, K. R., Claude, A., and Fullam, E., *J. Exp. Med.*, 1945, **81**, 233.

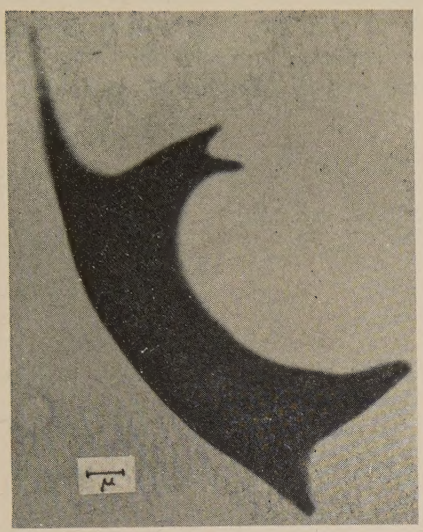

FIG. 2.

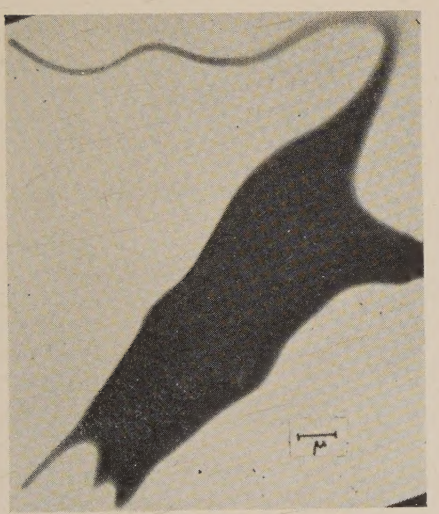

FIG. 3.

from which the disease receives its name and a rounded form with an unusually thin corpuscular center. Ordinarily normal red corpuscles present relatively dark centers with this technic. The decreasing hemoglobin content of the points of the sickled form is apparent.

Fig. 2 (× 5,000) is an electron micrograph of one of the multi-pointed forms which retains a suggestion of the characteristic crescent-like outline. One of the points has been transformed into an elongated structure.

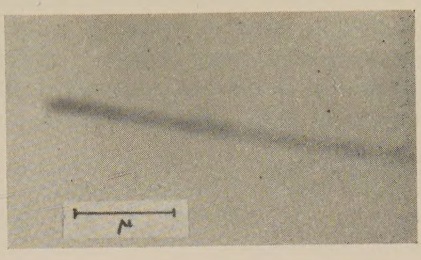

FIG. 4.

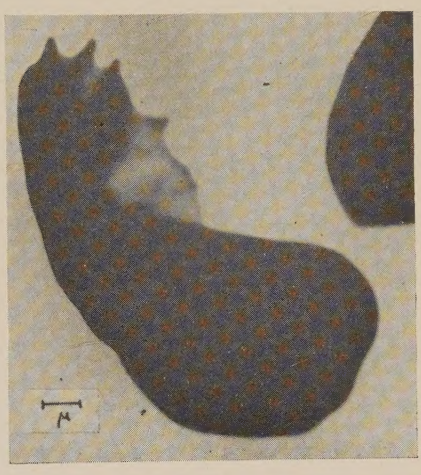

FIG. 5.

Fig. 3 (× 5,000) depicts another multi-pointed form with an elongated filament protruding from one pole.

Fig. 4 (× 13,000) is a higher power view of a portion of another but similarly elongated polar filament. The hemoglobin does not appear to be distributed homogeneously along the filament core.

Fig. 5 (× 5,000) depicts a stage intermediate in the development of the sickled form. At one pole of the cell elongation has progressed and numerous pointed processes are in evidence. These processes vary as to hemoglobin content. In the concavity of the crescent, hemoglobin has been withdrawn so that a large area presents the opposing corpuscular membranes in near approximation.

Further electron micrographs of developmental forms intermediate between disc- and crescent-shaped and/or multi-pointed forms will be the subject of a subsequent paper.

Summary. A modification of the Beck-Hertz technic permits direct mounting of sickle cells for electron microscopy studies. Electron micrographs of sickle cells essentially confirm the findings of Diggs and Bibb[2] and substantiate pertinent optical studies as to structural detail. In addition, both corpuscular hemoglobin-membrane relationships and polar filament structure are further visualized.

(Continued from page iii)